Chemical and Biological Engineering

BIOTECHNOLOGY APPLICATIONS OF MICROALGAL INDUCED DEFENCES. A SCENEDESMUS SPECIE CASE STUDY

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To my Mother and Sister

The most brave, strong and inspiring women I have ever met and ever will.

DECLARATION

I, Sebastiana Roccuzzo, declare that I am the sole author of this thesis and that the research presented within is the result of my own efforts and achievements, unless otherwise acknowledged in the text. I confirm that this work has not been submitted for any other degree.

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ABSTRACT

The cultivation and downstream processing of microalgal biomass for low to medium value products has high associated costs (Uduman et al., 2010), and despite the emergence of new technologies and efforts to increase efficiencies, significant improvements for large-scale production are still required. Open raceway ponds represent the cheapest method of large-scale microalgae production, requiring only low power inputs and relatively simple maintenance (Vieira Costa et al., 2014). However, these systems still experience numerous limitations such as contamination risks from undesirable organisms, i.e. grazers, which could potentially damage the entire algal cultivation (Montemezzani et al., 2015). Also, commercialisation of a variety of algal bioproducts is still limited, namely due to high operating costs in downstream processing, with the most crucial and expensive step being dewatering and biomass harvesting, accounting for up to 30% of the overall production cost (Uduman et al., 2010, Vandamme et al., 2013).

Chemical cues released by grazers like *Daphnia* and know as *infochemicals* can induce defensive responses in microalgae, including colony formation, flocculation and other morphological changes (Hessen & van Donk, 1993, Lampert et al., 1994, Lürling & van Donk, 1996, Lürling, 2003). This thesis investigates this phenomenon, as a process which could be exploited within biotechnology to facilitate flocculation of algal cells and therefore harvesting. More specifically, the focus is on the green alga *Scenedesmus subspicatus* and the zooplanktonic organism *Daphnia magna,* which act as exemplar organisms.

This thesis main aim was to present a *Daphnia*-induced bioflocculation method to make the algal biomass harvesting process affordable and more sustainable. This raised the following core objectives:

- to assess the impact of the specificity of microalgae – grazers interactions and how these can be exploited within algal biotechnology;
- to experimentally evaluate the fundamental working parameters allowing a feasible and efficient bio-flocculation approach;
- to distinguish between colony formation and aggregation of algal cells to unravel which cellular responses contribute to flocculation;
- to evaluate whether the flocculation process is driven by the production of EPS (extra polymeric substances);
- to reveal major metabolic pathways altered by exposure to the infochemical cues and key to flocculation and EPS production via a proteomic approach.

These objectives were addressed in the six chapters which form this thesis. The literature review provided in Chapter I covered a variety of studies undertaken from an ecological perspective, as well as the more relevant and recent biotechnological viewpoint. This is followed by Chapter II, where a meta-analysis on existing data sets was undertaken to investigate patterns associated with the complex interactions between *Daphnia* grazers and the microalga *Scenedesmus.* As infochemicals may be highly species-specific and even strain specific, it was important to investigate any specificity as this could impact on strain selection for industrial biomanufacturing. Also, the effect size of grazer cues was estimated for the first time, allowing a standardized comparison among various *Daphnia* grazers. The meta-analysis

presented facilitated investigations into these mechanisms by synthesizing several metrics of colony size, including cell number and overall colony size. The work presented cut across several disciplines, data reporting methods, experimental conditions and importantly, the strain/genotype/species identity of grazer and algae, providing the first quantitative assessment of the importance of microalgae-grazers species-specific interactions (Roccuzzo et al., 2016). Key findings were related to a significant effect of grazer identity, an effect size similar, or even higher under certain conditions, than commercial surfactants and no differences related to algae strains. Interestingly, meta-analysis results showed how the poorly studied grazer *Daphnia pulicaria* could induce changes in *Scenedesmus* spp mean particle volume (defined as the ratio between the total algal volume (μm^3/ml) and the number of particles per ml (van Holthoon, et al.,2003)), which were not only higher than all other grazers under study, but generated these responses at very low culture densities (5-20 ind/L). Due to the small amount of data however, more research is required to investigate the performance of this grazer species on inducing microalgal bio-flocculation. Chapter III provided an experimental investigation of key parameters associated with flocculation including initial algal concentration and age of the culture, infochemicals dosage, flocs size and cell surface characteristics. Perhaps surprisingly, dose-response results indicated that algal growth rate was not affected by the *Daphnia* cues at any stage of the culture, and therefore a metabolic cost was not associated to this defensive response to predators. However, significant flocculation efficiency results could only be achieved for algal cultures at early exponential stage and exposed to the highest concentration of infochemicals (FE = 77%), while progressively decreasing for older cultures (FE = 44%). Colony formation was shown to be a distinct phenomenon from flocculation, since flocs were predominantly composed by unicells while total cultures

registered an increase in coenobia, i.e. 2-, 4- 8-celled colonies. Interestingly, the dose-response trend for flocculation efficiency was different from what was expected in the case of a charge-neutralization mechanism (a quadratic flocculation rate with increasing infochemicals dose, with efficiency lowest at high and low doses (Billuri et al., 2015, Guo et al., 2015)) or cell-cell adhesion process (linear increase with increasing infochemicals dose). Therefore, it was hypothesised that infochemical-induced flocculation in *S. subpsicatus* occurs upon response to a biochemical trigger, and a specific amount of infochemicals might be needed per algal cell to trigger the response. Another interesting result from this chapter was that algal cultures at stationary phase were dominated by 4-celled coenobia before exposure to any infochemicals dosage. While colonies increased in the total cultures after exposure to *Daphnia* cues, flocculation did not occur at this growth stage for any infochemicals dosage. Based on the previous results, it was concluded that while colony formation was the result of a cell division process producing binary multiples of cells connected by a common cell wall, flocculation was more linked to aggregation of unicells. As there was no indication of charge neutralization-like mechanisms but rather a biochemical stimulus, it was hypothesised that the flocculation process was driven by the production of EPS, either in higher amount or with different distribution of components (Chapter IV). Subsequently, the focus was on the assessment of sEPS (soluble EPS) of *S. subpsicatus*, and the abundance of sugars, proteins and uronic acids in the sEPS. In fact, the relative ratios of the EPS components can influence its hydrophobicity and therefore impact cells aggregation and flocculation (Quigg et al., 2016). Also, the presence in the EPS of uronic acids may facilitate flocculation, as their carboxyl groups provide effective sites for the attachment of cells (Zhong et al., 2014). Negative staining was preliminary used to visualise and compare planktonic cells

versus cells in flocs. sEPS were then extracted and subjected to standard assays for proteins, sugars and uronic acids. While microscopy images seemed to indicate the presence of EPS surrounding cells and accumulating in the inner part of the algal flocs, surprisingly, no significant difference in the amounts of any of the sEPS components under study was found between exposed and non-exposed algae. The only exception was represented by the "other" fraction, i.e. the difference between the total sEPS dry weight and the sum of the sugars/proteins/uronic acids amounts. Independent NMR-based analysis speculated this other fraction as "small molecules, remnants of lipid based materials". The role of EPS components on algal flocculation other than the most commonly studied proteins and polysaccharides is not well established yet, although their hydrophobic and/or hydrophylic features can considerably affect the process. The presence of the significant portion of the other fraction in the sEPS and in higher amount for *S. subspicatus* cells exposed to infochemicals suggests further investigations would be needed to unravel the eventual presence of lipids responsible for cells aggregation. sEPS production could account for inducing flocculation in *S. subspicatus*.

Omics approaches have been proposed and trialled to analyse pathways and functions linked to EPS production, flocculation and colony formation in microalgae and cyanobacteria (Prochnik et al., 2010, Gulez et al., 2014, Schmid et al., 2015, Yu et al., 2015, Khona et al., 2016, Harke et al., 2017). Here the focus was on the proteomic response of *S. subspicatus* to naturally occurring infochemicals from the herbivore grazer, *D. magna*. The main objective was to reveal major metabolic pathways (e.g. protein, lipid and carbohydrate synthesis, stress responses) altered by exposure to the infochemical cues and central to the formation of flocs and EPS production. The approach here used relied on quantitative proteomics (iTRAQ). Changes were

observed at early exponential stage of algal cells and at “alarm” and “acclimation” phases of the exposure to infochemicals. These sampling times were chosen to observe variations early enough under infochemicals effects and at a time after which no further flocculation was observed. Results indicated bio-flocculation of *S. subspicatus* in response to *Daphnia* infochemicals occur already at the alarm phase and requires increased energy resources; also, an important role was envisaged in the synthesis of cysteine, a primary amino acid, precursors of defense biomolecules and promoter of bio-flocculation through the production of extra-cellular proteins with disulphide bonds (Xie et al., 2013, Romero et al., 2014, Aziz et al., 2016, Shi et al., 2017). Higher abundance of proteins related to photosynthesis, coupled with decreased protein abundance for carbohydrates metabolism, suggested bio-flocculation is boosted by production of different molecules other than polysaccharides and which would constitute the EPS matrix responsible for holding algal cells together. The data also indicated infochemicals induced flocculation may be sustained through MAPK signalling cascades. As previously mentioned, it remained important to distinguish between flocculation and colony formation and the proteomic experimental results, contrasting floc and planktonic cell responses, supported this idea that there are indeed two separate processes. In fact, and in contrast to flocculation, colony formation required higher energy demands at the alarm phase which later decreased at the acclimation stage, therefore suggesting a trade-off between colony formation and support of floc form. Finally, results suggested a role of fatty acids metabolism in the process of colony formation, as they contribute to the several cellular functions, including the accurate separation of membranes during cell division (Haddaji et al., 2017). The final chapter summaries how the work undertaken in the thesis has progressed the overall concept of

exploiting nature's chemical cues, with suggestions on what future research would be required to advance it further towards application.

REFERENCES

1. Aziz M, Nadipalli RK, Xie X, Sun Y, Surowiec K, Zhang JL, Paré PW. Augmenting sulfur metabolism and herbivore defense in Arabidopsis by bacterial volatile signaling. Frontiers in plant science. 2016 Apr 8; 7:458.

2. Billuri M, Bonner JS, Fuller CB, Islam MS. Impact of Natural Cationic Polymers on Charge and Clarification of Microalgae Suspensions. Environmental Engineering Science. 2015;32(3):212-21.

3. Gulez G, Altintas A, Fazli M, Dechesne A, Workman CT, Tolker-Nielsen T, et al. Colony morphology and transcriptome profiling of Pseudomonas putida KT2440 and its mutants deficient in alginate or all EPS synthesis under controlled matric potentials. Microbiologyopen. 2014;3(4):457-69.

4. Guo J, Yu J, Xin X, Zou C, Cheng Q, Yang H, et al. Characterization and flocculation mechanism of a bioflocculant from hydrolyzate of rice stover. Bioresource Technology. 2015; 177:393-7.

5. Haddaji N, Mahdhi AK, Ben Ismaiil M, Bakhrouf A. Effect of environmental stress on cell surface and membrane fatty acids of Lactobacillus plantarum. Archives of Microbiology. 2017;199(9):1243-50.

6. Harke MJ, Jankowiak JG, Morrell BK, Gobler CJ. Transcriptomic Responses in the Bloom-Forming Cyanobacterium Microcystis Induced during Exposure to Zooplankton. Applied and Environmental Microbiology. 2017;85(4):e02832-16.

7. Hessen DO, Vandonk E. Morphological changes in Scenedesmus induced by substances released from Daphnia. Archiv Fur Hydrobiologie. 1993;127(2):129-40.

8. Khona DK, Shirolikar SM, Gawde KK, Hom E, Deodhar MA, D'Souza JS. Characterization of salt stress-induced palmelloids in the green alga, Chlamydomonas reinhardtii. Algal Research-Biomass Biofuels and Bioproducts. 2016; 16:434-48.

9. Lampert W, Rothhaupt KO, Vonelert E. Chemical Induction of colony formation in a green-alga (Scenedesmus acutus) by grazers (Daphnia). Limnology and Oceanography. 1994;39(7):1543-50.

10. Lürling M, Van Donk E. Zooplankton-induced unicell-colony transformation in Scenedesmus acutus and its effect on growth of herbivore Daphnia. Oecologia (Berlin). 1996;108(3):432-7.

11. Lürling M. Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. Annales De Limnologie-International Journal of Limnology. 2003;39(2):85-101.

12. Montemezzani V, Duggan IC, Hogg ID, Craggs RJ. A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. Algal Research-Biomass Biofuels and Bioproducts. 2015; 11:211-26.

13. Prochnik SE, Umen J, Nedelcu AM, Hallmann A, Miller SM, Nishii I, et al. Genomic Analysis of Organismal Complexity in the Multicellular Green Alga Volvox carteri. Science. 2010;329(5988):223-6.

14. Quigg A, Passow U, Chin WC, Xu C, Doyle S, Bretherton L, et al. The role of microbial exopolymers in determining the fate of oil and chemical dispersants in the ocean. Limnology and Oceanography Letters. 2016;1(1):3-26.

15. Roccuzzo S, Beckerman AP, Pandhal J. The use of natural infochemicals for sustainable and efficient harvesting of the microalgae Scenedesmus spp. for biotechnology: insights from a meta-analysis. Biotechnology Letters. 2016;38(12):1983-90.

16. Romero LC, Angeles Aroca M, Laureano-Marin AM, Moreno I, Garcia I, Gotor C. Cysteine and Cysteine-Related Signaling Pathways in Arabidopsis thaliana. Molecular Plant. 2014;7(2):264-76.

17. Schmid J, Sieber V, Rehm B. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Frontiers in microbiology. 2015 May 26; 6:496.

18. Shi J, Chen Y, Xu Y, Ji D, Chen C, Xie C. Differential Proteomic Analysis by iTRAQ Reveals the Mechanism of Pyropia haitanensis Responding to High Temperature Stress. Scientific Reports. 2017;7.

19. Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy. 2010;2(1):012701.

20. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

21. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology. 2013;31(4):233-9.

22. Vieira Costa JA, de Morais MG. An Open Pond System for Microalgal Cultivation. Biofuels from Algae. 2014:1-22.

23. Xie GJ, Liu BF, Xing DF, Nan J, Ding J, Ren NQ. Photo-fermentative bacteria aggregation triggered by L-cysteine during hydrogen production. Biotechnology for biofuels. 2013 Dec;6(1):64.

24. Yu W, Chen Z, Shen L, Wang Y, Li Q, Yan S, et al. Proteomic profiling of Bacillus licheniformis reveals a stress response mechanism in the synthesis of extracellular polymeric flocculants. Biotechnology and Bioengineering. 2016;113(4):797-806.

25. Zhong C, Xu A, Chen L, Yang X, Yang B, Hong W, et al. Production of a bioflocculant from chromotropic acid waste water and its application in steroid estrogen removal. Colloids and Surfaces B-Biointerfaces. 2014; 122:729-37.

CHAPTER I

Overcoming the challenge of Scenedesmus harvesting: concepts from a combination of Industrial and Synthetic Ecology.

1.1 Introduction

Algal organisms are photosynthetic macro- or micro-algae which grow in terrestrial or aquatic habitats. Macroalgae are multicellular plants able to grow fast in either fresh or salt water. Based on their pigmentation they are classified in brown (Phaeophyceae), red (Rhodophyceae) and green (Chlorophyceae) (Demirbas and Demirbas, 2011). Microalgae are microscopic organisms which can be found in both freshwater and marine enviroments as well as terrestrial surfaces. They are classified according to their colour, life cycle and cellular structure. The three most important classes, in terms of their abundance, are diatoms, green algae, and golden algae. Cyanobacteria, or blue-green algae, are also referred to as microalgae (Demirbas and Demirbas, 2011). There are about 80,000 to 100,000 different algal species with size ranges from micrometres (microalgae) to tens of metres (macroalgae) (Enzing et al. 2014). The organisms considered in this thesis are microalgae growing in freshwater environments. There is a growing interest worldwide on algae as cell factories, as they contain lipids, proteins, carbohydrates and pigments which can be marketed as food, feed supplements, fertilisers, cosmetics and much more (Sharma & Sharma, 2017) (Fig. 1-1).

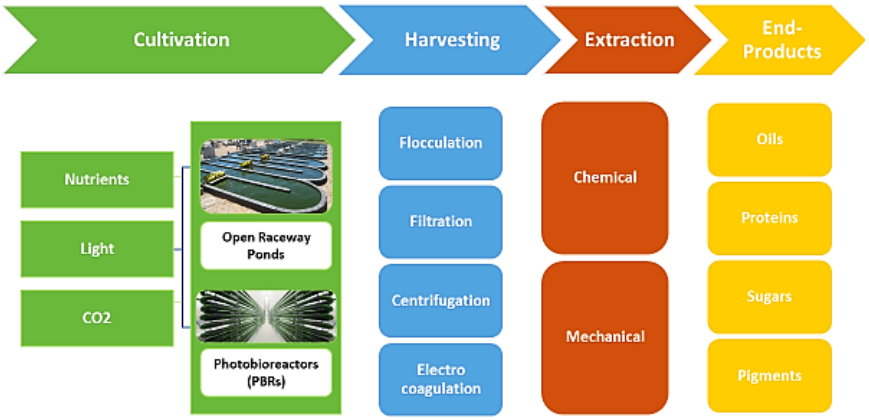


Fig.1-1 Diagram of production cycle and possible products obtainable from algal biomass

Commercial large-scale cultivation of the microalgae *Chlorella* spp can be dated back to 1950s, followed by *Spirulina* in 1960s. 1980s saw the rise of large-scale facilities in Asia, India, United States, Australia, Israel to produce algae for food, feed, extraction of metabolites (Habib et al. 2008). More recently algae are being considered also for the bioethanol or biodiesel production (Tang et al., 2016). Research on genetically modified algae are on-going for the pharmaceutical sector (Demirbas and Demirbas, 2010, Enzing et al., 2014,).

Microalgae are sustainable commodities as they can be grown on non-arable land and wastewater for nutrients. They have microscopic dimensions; therefore, they can grow much faster than terrestrial crop plants; allow higher yields as well as reduced production costs, especially in integrated bioprocesses with CO_2 deriving from exhaust fumes and gases (Sharma & Sharma 2017). The identification of suitable microalgae strains is usually the very priority in the development of a microalgae-based technology; they should have high light capture efficiency as well as a high biomass yield, both in terms of growth rate and culture density, and high light intensity and oxygen concentration tolerance (Moreno-Garcia et al., 2017). Large flocculation properties would be also useful to facilitate harvesting, along with structural features allowing easy intracellular products extraction. The ideal strain should also present resistance to predators and grazers as well as other contaminants and efficiently use Nitrogen and Phosphorous but also the ability to use alternative sources of these macro elements (Ortiz-Marquez *et al.,* 2013). However, there are scientific and technological barriers to overcome before bulk goods from microalgae becomes an economic process; although some companies are already developing businesses of algal bio-products, there is still a great controversy among specialists about their actual potential (Scott et al., 2010). At present, not one of the suitable strains

identified for large scale production owns all the ideal traits mentioned, probably because, differently to the development of modern plant crops, a systematic breeding program for algae has never been realized (Ortiz-Marquez et al., 2013). Another important factor could be represented by the lack of solid knowledge on scaling-up techniques from successfully laboratory results to large-scale industrial applications (Shurin et al., 2013). Currently, optimal improvement of the desired properties or functions has been much more focused on the use of genetic engineering, also facilitated by the continuous improvements in genome-sequencing techniques (Georgianna and Mayfield, 2012). The efforts in this context have been mainly directed to the modification of genomes and cellular metabolism to increase cellular lipid concentrations, biomass productivity and resistance to predators. However, no modified strains have been authorised for outdoor cultivations (Shurin *et al.,* 2013). In addition, the associated environmental risks with genetic manipulation, although they are likely to be insignificant, are virtually unknown at present and thorough ecological and evolutionary assessments are still needed to test genetically modified algae can survive in the wild and their persistence cause environmental harm (Snow *et al.,* 2012).

1.1 Synthetic and Industrial Ecology Applied to Microalgal Biotechnology

The use of algal ecology principles has been reported to have the potential to lead to more stable open microalgal cultivation systems, disclosing practices that could be used to preserve and improve algal culture techniques and management (Kazamia *et al.,* 2012). In the context of biotechnology applications, a synthetic ecology approach combined with industrial ecology design might allow to overcome some of the trade-offs related to performance of microalgae functions. In fact, while synthetic ecology implies the application of engineering principles to biology and the rational synthesis

of targeted, complex systems where the building blocks are cells in a mixed community (Pandhal and Noirel, 2014), industrial ecology, a discipline which describes the analogy between industrial and natural systems to promote the development of sustainable industrial practices, has the potential to improve total environment quality while complying with economic demands of industry, providing the tools for improvement of existing production processes as well as supporting policies to boost innovation and commercialisation of new and improved products making use of surplus materials, water and energy (Jelinksi et al., 1992, Tibbs, 1993, Erkman, 1997, Roberts, 2004). A combined synthetic-industrial ecology procedure could offer many important advantages, such as the possibility to isolate specific strains from their natural habitat for studies in a more favourable and defined artificial context, allowing to predict how the algal community might develop and consequently optimise the algae cultivation systems for a specific goal (Rollie' et al., 2012, Kazamia et al., 2012). Re-designing natural ecosystems as well as unravelling molecular pathways rather than "simply" modifying the genomes of individual organisms or species, as it is instead for genetic engineering, could also lead to several important practical applications, such as the utilization of metabolic potential of organisms that may be difficult to genetically modify.

1.3 Large Scale Cultivation of Microalgae

As the global need for bioproducts is rising, microalgae are increasingly seen as part of the solution to meet increasing demands, thanks to the great diversification of products that can be obtained from microalgal biomass, such proteins, glycerine, pigments, nutraceuticals and fuels (Jena & Hoekmann, 2017). Microalgal biomass has found several industrial applications in areas like dietary supplements, lipids, biomasses, pigments, fertilizers and bio-fuels. For these purposes, microalgae can be

grown using CO_2 and industrial wastes, so reducing the cost of culture nutrients and mitigate the environmental issues related to these effluents (Vieira Costa *et al.,* 2011, Sutherland and Craggs, 2017), as they can accumulate nutrients, heavy metals, pesticides, as well as organic and inorganic toxic substances or even radioactive elements in their cells (Sen et al., 2013). Nonenetheless, the production of microalgae biomass has still high costs, especially if compared to more "traditional" agricultural and forestry biomasses, so representing a major issue in the achievement of an economically viable industrial manufacturing process (Acien et al., 2012, Ruiz et al., 2016). Despite several attempts of process optimization, the development of cultivation systems being both cost-effective and highly efficient still need to be significantly improved for large-scale production to become attainable (Rizwan et al., 2015, Lammers et al., 2017). While data has been generated at a laboratory-scale, not much has been published in way of technology transfer to large scale (Rawat et al., 2013). Phototrophic cultivations appear a favoured method for algae cultivation, as the sunlight is freely and abundantly available. Also, phototrophic algae can capture carbon dioxide from exhaust gases, so potentially acting as a superior carbon sink (Lam et al., 2012). This method however presents some weaknesses, especially in those temperate regions where suitable sunlight intensity is not always available throughout the year. Both open ponds and closed photobioreactors are suitable for the cultivation of phototrophic algae. In any case, an ideal system should meet at least one requirement amongst availability of a large effective illumination area, optimal gas-liquid transfer, simple management, low contamination level, low capital and investment costs or minimal land requirements.

The following section details the basic design, the main advantages and limitations as well as the factors to be considered before attempting a scale-up, of the cultivation

systems currently used, photo bioreactors (PBRs) and open ponds, with a special emphasis on the latter. Although, there are advantages and disadvantages associated with using both, here the focus is on open raceway ponds production systems, as they represent the cheapest method of large-scale micro-algal production for low-medium value products, requiring only low power inputs, easy maintenance and cleaning (Vieira Costa *et al.,* 2013).

1.3.1 PHOTOBIOREACTORS

This type of production system is mainly considered when the main interest is towards the production of high value products, i.e. pigments, food additives for human consumption, proteins etc. Even in this case however there is still a need for cost-effective PBRs that can overcome the initial investment issues and at the same time provide large scale efficient cultivations. Compared to open ponds, closed photobioreactors may show higher photosynthetic efficiencies and biomass production as well as a degree of control. However, they require high initial cost and only microalgal strains with specific physiologies may be employed (Vieira Costa *et al.,* 2014). It has also been reported that PBRs can experience problems with virus susceptibility and/or bacteria attacks, which can completely crash the production system down in a few hours. In the last decades, different types of closed photobioreactors have been developed, such as flat plate, tubular and column, stirred mechanically or by airlifting. However, these systems are limited by the excess of oxygen being produced and their cost is generally high. The use of sterile systems allows controlling contamination, but, on the other side, this lead to a cost increase. Moreover, the scale up of PBRs generally requires an increase of the tube's diameter, so preventing cells to receive adequate light for their growth (Vieira Costa *et al.,* 2014). The main feature of a photobioreactor influencing algal exposure to light is the

Surface/Volume ratio. Some of the materials used for constructions of reactors are glass, Plexiglas, PVC, acrylic-PVC and PE. An important characteristic of the material to be employed is its ability to prevent biofilm formation. In fact, although biofilms can be easily cleaned, they can dramatically decrease light transmission.

1.3.2 Simple Ponds

Operation is very simple for these systems, having only a giant rotating mixer at the centre of the pond to avoid precipitation of algal biomass. However, they show a major disadvantage represented by the surrounding environment which cannot be entirely controlled in terms of temperature or light availability (Yen *et al.,* 2013). Moreover, contamination from bacteria or other microorganisms often results in the predominance of undesirable species. Rainy conditions also represent a common source of contamination. Consequently, the selection of an appropriate location is crucial to the success of such systems. Despite the potential related drawbacks with the simple open pond systems, their ease of operation and high scale-up availability still represent attractive factors and they are currently used for industrial production (Yen *et al.,* 2013).

1.3.3 Raceway Pond Systems

Currently the most economical cultivation method for large-scale algal biomass production is represented by the raceway pond system, mainly due to its relatively low capital cost as well as ease of operation and management. The pond is usually made up of an oval-shaped closed loop recirculation channel, where paddlewheels provide mixing and circulation, so ensuring the homogenization of culture and consequently stabilization of algal growth and productivity (Fig. 1-2) (Vieira Costa *et al.,* 2014).

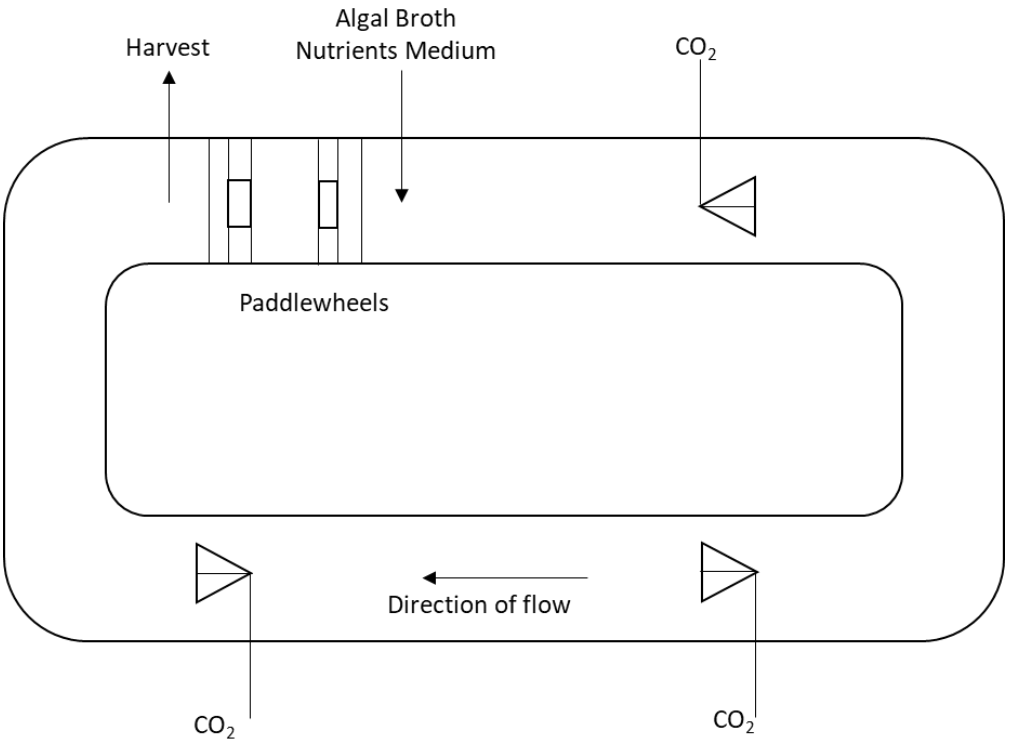


Fig. 1-2 Plan view of a raceway pond. Nutrient input is inserted after the paddlewheel and executes a cycle while being aerated with Carbon Dioxide. It is harvested before the paddlewheel to begin another cycle (adapted from Brennan and Owende, 2009).

Some raceway ponds include artificial light in the system but this method is neither practical nor cost-effective for commercial production. Raceway ponds can be constructed in several materials like concrete and compacted earth and lined with plastic bags. Ponds are shallow with a depth usually in the range 20-50 cm to ensure an adequate sunlight exposure to algae (Lam *et al.,* 2014). Despite the several advantages offered by raceway ponds, the foremost of which being low energy input and low operating cost, this system can still experience numerous limitations, like high harvesting costs, water loss caused by high evaporation rate, easy contamination by unwanted organisms, i.e. grazers, bacteria, fungi and protozoa, that could potentially "crash" or collapse the entire algal population. Moreover, it is difficult to control parameters like temperature and pH. Contamination of cultures by different species of microalgae in open pond systems is controlled by effectively operating them as batch

cultures and restarting the cultures at established intervals with new water and mono-algal inoculum. Contamination from insects, leaves and airborne materials must be controlled within acceptable limits as well. In open ponds, these contaminants are regularly removed by using, manually or automatically, a sieve in the water flow. However, if the microalgal biomass is applied to products like biofuels, impurities are acceptable in the cultivation (Vieira Costa *et al.,* 2014).

1.4 Harvesting Challenges

Although algal based manufacturing is technologically feasible, its wide marketing is still limited because of high operating costs in processing. Four main steps are required for bioproducts production from microalgae biomass: cultivation, harvest, extraction of compounds of interest and processing. The most crucial and expensive stages for low-medium values bioproducts have been identified in harvesting and dewatering steps, as they require high energy inputs for separation of biomass from a dilute culture medium, accounting for around 20-30% of the overall production cost (Lee *et al.,* 2013). Hence, their efficiency, versatility, productivity and recovery optimization should become a priority for obtaining cost effective viable algae-based products. For production purposes, microalgae should be concentrated as much as possible so allowing the reduction of the subsequent drying process as well as extraction and purification costs. Furthermore, contaminant or toxic de-watering processes should be avoided for water medium recycling to be possible (Uduman *et al.,* 2010). Harvesting of microalgae requires the concentration of dilute suspensions, average compositions in the range 0.02% - 0.06% Total Suspended Solids (TSS), into slurry or paste with 5%-25% TSS or more, based on the process main goal. Surface charge, steric effects and adsorbed macromolecules or extracellular organic matter are the main factors influencing microalgae stability. Unlike other types of suspended

particles, microalgae consist of different species with diversified properties such as shape, size and motility, each of which affects their reactions to treatment (Uduman *et al.,* 2010). As a result, despite the development of several techniques for microalgae culture dewatering and harvesting, no one performs better than all the others. Existing processes rely upon the improvement of suitable properties which facilitate harvesting and dewatering and increase their efficiency; among them, we can mention a) large cell size, b) higher specific density than the medium, and c) autoflocculation or induced flocculation. Quantitative performance assessment relies on the evaluation of the rate of water removal, solid content of the recovered microalgae-water slurry and efficiency of dewatering technique, i.e. recovered microalgae to total processed microalgae, through measurements of absorbance and/or turbidity.

1.4.1 Harvesting by Flocculation

This thesis is focused on microalgae harvesting by flocculation, as it is generally considered the most economical method for the treatment of high volumes of microalgae cultures and its application to a broad range of species (Uduman *et al.,* 2010). In general, an algal cell can be viewed as a very tiny spherical object, falling in a continuous viscous medium at a rate governed by the force of gravity and the upward drag and buoyancy forces. In theory, if the algal particle moves in the fluid by its own weight due to gravity then it reaches a settling velocity when the combined drag and buoyancy force, exactly balances the force of gravity (Stokes' law). However, the settling velocity of an algal particle in a natural context is controlled by several complex factors, including cell mobility, water flow and turbulence as well as upwelling caused by wind and/or temperature stratification. For planktonic algae, settling velocity can be increased by enhancing cell dimensions, for example inducing cell aggregation into a larger body. This principle is applied in the processes of algae

separation, where nowadays chemical coagulants are added to form large flocs which quickly settle to the reactor bottom (Show *et al.,* 2013). The coagulants and flocculants commonly used consist of metal salts such as poly-aluminium chloride and alum as well as synthetic polymers like polyacrylamide, as they are reliable and efficient (Alam et al., 2016). However, the use of these chemicals may have several environmental consequences, first of which the contamination of the produced biomass, an increase in metal concentration in water and the production of large volumes of potentially toxic sludge (Renault *et al.,* 2009). Biopolymers like chitosan are also alternatively used to avoid biomass contamination; however, these are currently too expensive for low-value compounds manufacturing. Other technologies like electrocoagulation have proven to be efficient, non-exempt however from metal contamination, since the electricity flowing through the medium causes more metal to be dissolved and form ions (Marrone et al., 2017), or high energetic costs at scale (Alam et al., 2016),

Flocculation of microalgae can also be induced by several microorganisms, such as bacteria or fungi (Lee et al., 2013, Manheim and Nelson 2013, Muradov et al., 2015) by extra polymeric substances (EPS) (Jakob et al., 2016, Busi et al., 2017), and it is often referred to as bio-flocculation (Vandamme et al., 2013). Bio-flocculation of microalgae is influenced by various factors, i.e. nutrients status, pH, algal species which make it a complex process to control and still hinder its application at scale. Nonetheless, being a potential low cost, non-toxic, metal-free harvesting method it has a great potential for the manufacture of low-medium value compounds and therefore gaining a rising attention in the field (Alam et al, 2016). Other than bacteria or fungi, infochemicals are starting to be explored as potential bioflocculants (Vandamme et al., 2013, Montemazzani et al., 2015, Alam et al., 2016, Roccuzzo et al., 2016, Zhu et al., 2017).

1.4.1.1 An Overview of Coagulation and Flocculation Theory

For practical purposes, precipitates and particles classification as suspended or colloidal depends on their size range. In particular, suspended particle size spans from 0.1 µm up to 100 µm, while colloids are in the size interval between dissolved substances (0.001 -0.1 µm) and suspended particles. Some examples are reported in Fig. 1-3.

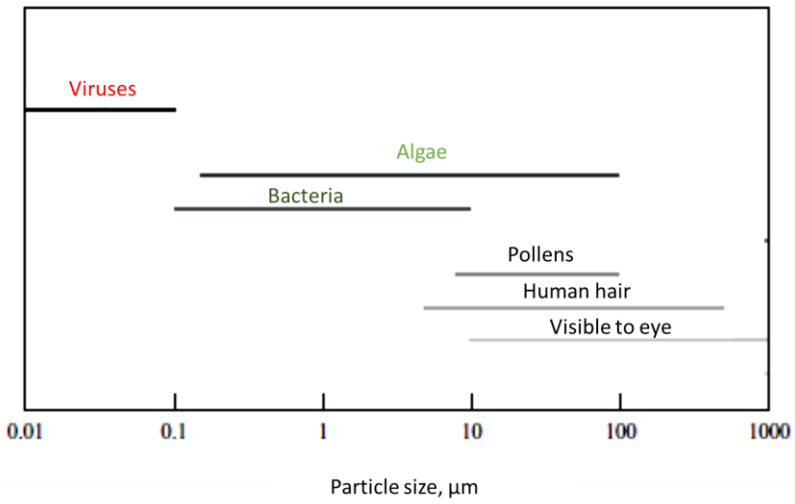


Fig. 1-**3** Particulates in water and various other reference sizes (adapted from Davis 2010)

Colloidal particles are in a solid state and can be removed from the liquid by physical means such as very high-force centrifugation or filtration; their small size however prevents their removal by sedimentation or sand filtration processes. Fundamentally, the goal of coagulation, and subsequently flocculation is the conversion of small particles into larger ones called flocs, either as precipitates or as suspended particles, whose ready removal can take place in subsequent processes, such as settling or filtration. In this context, we refer to coagulation as the process of chemical addition, while to flocculation as the aggregation process of the destabilized particles and precipitation products (Davis 2010).

1.4.1.2 Particle Characteristics

1.4.1.3 Electrical properties

Surface charge of colloidal and suspended particles represents their most important electrical property, as it keeps them in suspension, preventing their aggregation for long periods. Particle suspensions are thermodynamically unstable and, given enough time, they will flocculate and settle (Davis 2010). This process however is slow-paced, so precluding a feasible removal of particles by sedimentation. Most particles in water are negatively charged, mainly because of processes like ionization, adsorption and structural imperfections. Microalgae cells have a net negative surface charge due to the ionization of functional groups and the stability of their suspensions relies upon the forces interacting between the particles themselves and the particles and the surrounding medium (water) (Uduman et al., 2010).

1.4.1.4 Electrical Double Layer

A colloidal dispersion in solution does not have a net charge as the negatively charged particles gather positive counter ions on and near the particle surface, so forming a double layer (Fig.1-4). The adsorbed layer of cations, known as the Helmholtz or Stern layer, has a thickness of about 0.5 nm and it is bound to the particle surface by electrostatic and adsorption forces. A loose diffuse layer forms beyond the Helmholtz layer, and the resulting double layer (Helmholtz plus diffuse) has a net negative charge over the bulk solution, whose extension depends on the solution properties (Davis 2010).

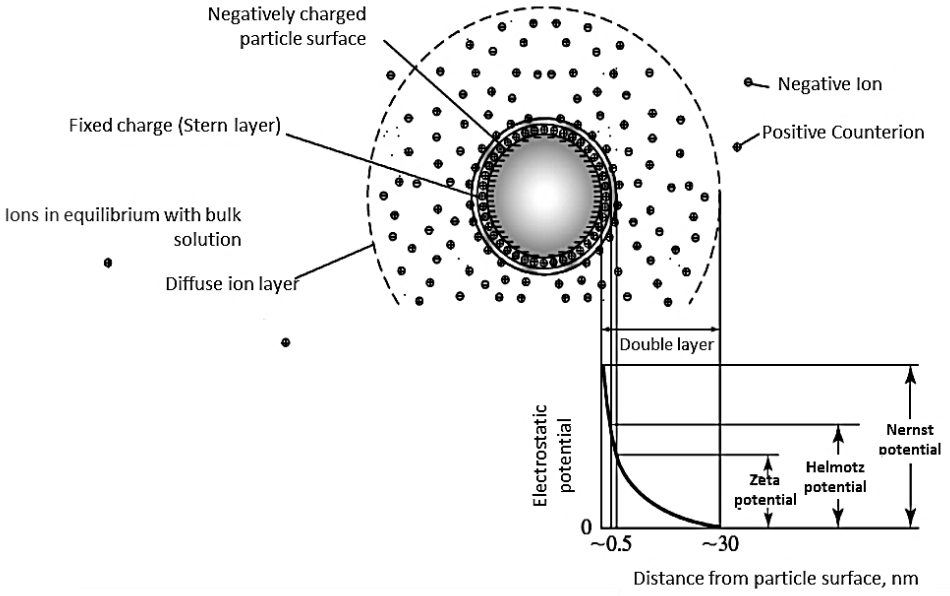


Fig. 1-**4** Surface charge on a particle in water (adapted from Davis, 2010)

1.4.1.5 PARTICLES STABILITY

The electric potential between the shear plane and the bulk solution is known as the *zeta potential*. Empirically, rapid flocculation takes place when the absolute value of zeta potential is reduced below 20 mV (Kruyt, 1952). Particles stability in natural waters is described by the DLVO theory (Derjaguin 1934; Derjaguin and Landau 1941; Verwey and Overbeek 1948) and depends on the balance between the electrostatic force of the charged particles and attractive forces (van der Waals). As the particles have a net negative charge, the major mechanism regulating stability is the electrostatic repulsion. The double layer extends further into solution than the van der Waals forces, resulting in the generation of an energy barrier that prevents particles aggregation (Davis, 2010) (Fig. 1-5). The strength of van der Waals forces depends on the size and shape of the colloidal particles as well as the chemical composition of the system under study (Liang et al., 2007).

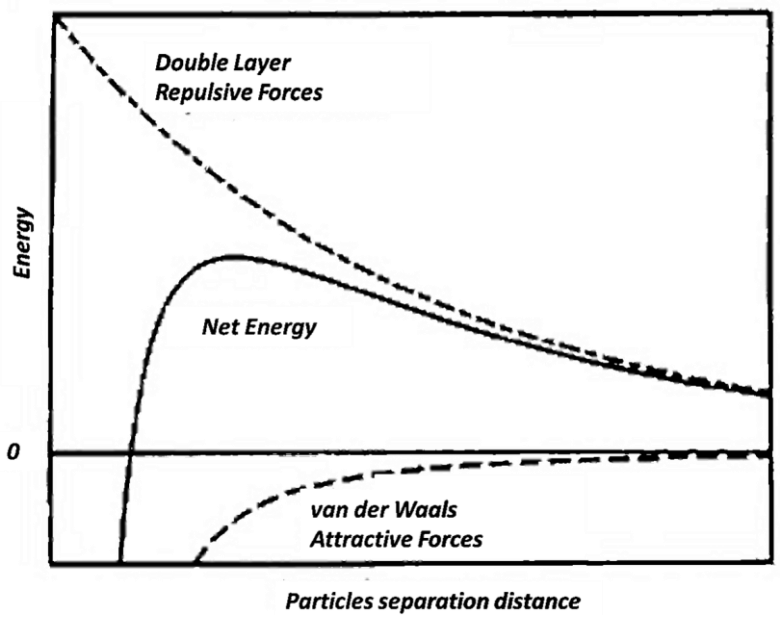


Figure 1-5 Schematic Representation of the DLVO theory

1.4.1.6 COAGULANTS

Several inorganic chemicals have been tested for microalgal flocculation and the most effective resulted to be alum, ferric chloride and certain cationic polymers such as polyacrlyamides and polyamines (Uduman *et al.,* 2010). Surface charges neutralization, a state where the net electrical charge of the microalgal particle has been annulled due to adsorption of an equal amount of the opposite charge, is the mechanism reported for microalgae flocculation by inorganic coagulants; its success mainly depends on the presence of small and approximately spherical algal particles.

Microalgal flocculation mechanism induced by polyelectrolyte flocculants, which are composed by natural or synthetic cationic species, can be explained by a combination of charge neutralization and particle bridging, the extent of which depending on charge density and polymer chain length. Coverage level of microalgal surface influences the degree of flocculation; in fact, for less than the optimum coverage value, an inadequate bridging (unable to withstand shear forces due to any agitation)

will occur. Conversely, an excessive coating causes electrostatic or static hindering of bridging. Concentration and reactivity of functional groups on microalgae cell walls greatly vary with growth phase and metabolic conditions, resulting in variation of their charge density and so affecting the adsorption of both organic polyelectrolytes and inorganic flocculants (Uduman *et al.,* 2010). Finally, combined flocculation is a process involving the use of more than one type of flocculants for its overall performance improvement and reduction of required dosages.

1.4.1. 7 pH and Dose

Two fundamental parameters in coagulants addition are pH and dose. Because of the number and complexity of coagulant reactions, the actual optimum dose and pH for given samples on a given day is generally determined empirically from a laboratory jar test. Generally, it is reported that the dose of required flocculant depends on microalgae surface area (Bleeke et al., 2015), which in its turn is influenced by their concentration, composition, surface charge density, charge density of the cationic flocculant as well as flocs size and density. One of the major disadvantages of using metal salts as flocculants for microalgae recovery is the addition of chemicals into the system, which impacts the environmental sustainability of the process. There is also a risk of potential contamination of the medium, preventing its recycling, and the resulting algal biomass therefore leading to a more complex downstream processing (Muylaert et al., 2017).

1.4.2 INFOCHEMICALS INDUCED FLOCCULATION

Infochemicals are substances excreted by organisms that may change the behavior, physiology and structure of individuals of another species (Ha et al., 2004). They can induce defense mechanisms in microalgae against zooplankton grazing by promoting colony formation or bio-flocculation (Hessen and van Donk*.,* 1993, Lampert 1994, Lürling, 1999, Lürling 2003). Some types of these infochemicals have been isolated and identified from *Daphnia spp*, being likely aliphatic sulfates and sulfamates (Fig. 1-6) (Yasumoto *et al.,* 2008.).

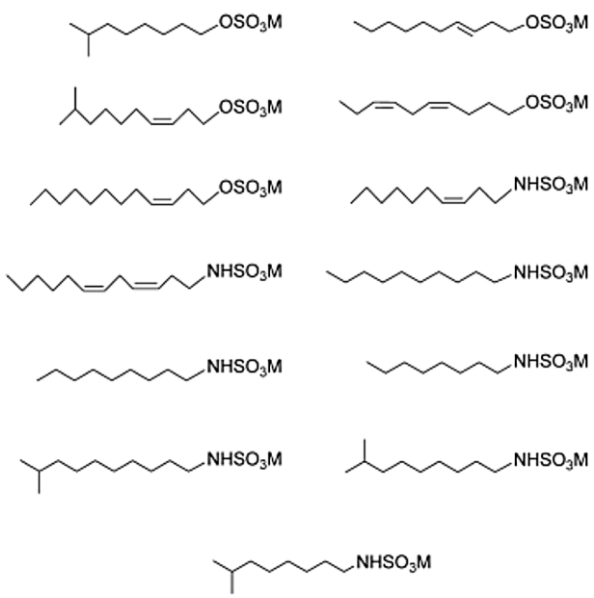


Fig. 1-6 Proposed structure of *Daphnia's* infochemicals, based on spectroscopic and synthetic studies of fractions extracted with organic solvents from frozen *Daphnia pulex* and reported to induce colony formation on the microalga Scenedesmus *gutwinskii var. heterospina* at ng—µg/ml concentration. M = not identified countercations (Yasumoto et al., 2008)

A summary of the current literature reports on infochemicals characterization work is reported in Table 1-1.

Table 1-1 *Infochemicals properties*

PRODUCER	RECEIVER	PROPERTIES	REFERENCE
Daphnia sp	*Scenedesmus* sp.	<0.5 kDa; insensitive to proteases; heat and pH stable; non-volatile; sensitive to incineration	Lampert et al., 1994
Daphnia sp	*Scenedesmus* sp.	Lipophilicity increased at low pH; olefinic double bonds; insensitive to sulphatase, phosphatase and proteases; Not free fatty acids	Von Elert et al., 1999
Daphnia sp	*Scenedesmus* sp.	Non-volatile	Van Hoolton et al., 2003
Daphnia sp	*Actinastrum* sp.	Not butanoic acid, acetic acid or amino acids	Yasumoto et al., 2005
Daphnia sp (homogenates)	*Scenedesmus* sp.	Aliphatic Sulfates and Sulfamates	Yasumto et al., 2005 and 2008
Daphnia sp	*Scenedesmus* sp.	Anionic Surfactants	Yasumoto et al., 2005
Daphnia sp	Green algae	8-methylnonilsulfate Sulfates Amidosulfates	Uchida et al., 2008

In large scale open raceway ponds, infochemicals could be potentially used to promote flocculation inducing defensive morphological changes in microalgae. The direct addition of purified biological infochemicals or extracts could represent an "easy" option to flocculate microalgae, however it would be necessary to account for their additional production and purification cost. On the other hand, these could be decreased considering a production system of infochemicals integrated in the microalgae cultivation site. In fact, as infochemicals are expected to be copious in open raceway ponds, the outflow coming from these could be filtered to remove the grazers and then recirculated into the system to boost colony formation and flocculation (Fig. 1-7).

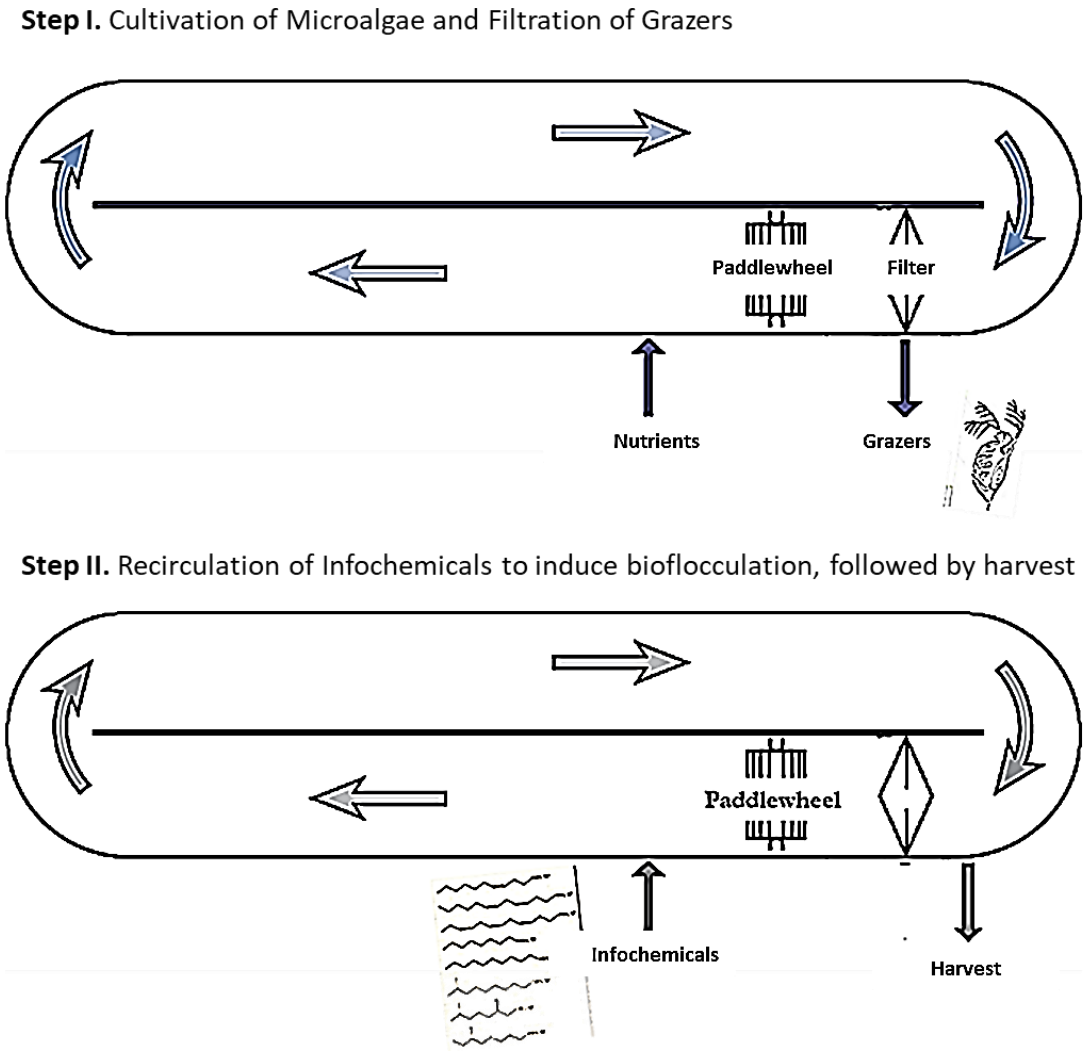


Fig. 1-7 Schematic representation of potential application of naturally occurring zooplankton infochemicals in open raceway ponds, per Industrial Ecology principles

Controlled flocculation of microalgae through infochemicals is a promising technology; however, the use of infochemicals is also likely to be highly species-specific. The underlying mechanism is still poorly understood and would deserve further research because it may lead to a metal-free method for flocculating microalgae. Fundamental research into infochemicals that induce flocculation in microalgae is urgently needed, because this may lead to a highly controllable method that avoids metals

contamination (Vandamme *et al.,* 2013). An efficient, sustainable method for harvesting microalgae is vital for affordable production of microalgal biomass on an industrial, commercial scale. The regulated use of naturally occurring infochemicals would allow a perfect combination of synthetic and industrial ecology principles, as it has the potential to maximize the use of resources, minimize waste generation and reduce energy costs.

1.5 Other Harvesting Methods

To date, the main harvesting techniques used other than flocculation include centrifugation, biofilms formation, filtration, flocculation, gravity sedimentation, dissolved air flotation, ultrasounds and electrophoresis techniques (Alam et al., 2016).

1.5.1 Centrifugation

This method allows rapid and efficient recovery of a very concentrated algal biomass; however, it is energy intensive and requires high cost for maintenance (Lam et al., 2012).

1.5.2 Floatation

This method is used in combination with flocculation and consists in the trapping of algal biomass by dispersing micro air bubbles, which adhere to the biomass, increase its buoyancy and hence causing its quickly rise to the surface. Its main advantage is represented by the applicability to large culture volumes; on the other hand, toxicity of flocculants might reduce the value of the biomass obtained (Lam et al., 2012). Other factors limiting a more extensive use of floation technology include the cost of equipment and energy efficiency of microbubbles production (Zimmermann et al., 2011). More recently, it has been proposed an alternative, potentially cheapest and

low maintenance production method which involves the use of a fluidic oscillator (Zimmermann and Tesar, 2013, Rehman et al., 2015).

1.5.3 Filtration

In this case, filter press and membrane filter are operated under pressurized or vacuum condition. Filter press method is very effective in algae recovering, especially for species of relatively large size but, for the same reasons, it cannot be used to recover small-sized algae, such as *Scenedesmus spp*. Micro/ultrafiltration are effective for recovering both large and small sized algae but they have also high costs mainly due to membrane replacement, clogging and maintenance (Lam et al., 2013).

1.5.4 Gravity Sedimentation

This method is very low cost as no additional chemicals and/or physical treatments are necessary but it takes relatively longer settling times and at the same time it is unfeasible for recovery of small algae cells.

1.5.5 Ultrasonication

The process relies on the use of ultrasound waves which propagate into the liquid media resulting in alternating high-pressure and low-pressure cycles. During the low-pressure cycle, high-intensity small vacuum bubbles are created in the medium while during the high-pressure cycle bubbles collapse violently in a process known as cavitation (Lee et al., 2014). During implosion, very high pressures and high-speed liquid jets are generated locally and the resulting shear forces immediately break the algal cell structure, hence facilitating sedimentation rate (Lam et al., 2013). The process can be operated continuously but it has also safety related issues to be accurately evaluated.

1.6 Open Raceway Ponds as Cultivation Vessels

Most of current commercial cultivation practices for algae rely on the use of open ponds, as they are cheap and with simple design and maintenance requirements. The factors governing algal biomass productivity are both biotic and abiotic, being mainly represented by nutrients supply, light, temperature, losses due to grazers, hydrodynamics of the reactor, CO_2 fixation, pH and sterility of cultivation. These can considerably vary on local environmental conditions, influencing their species composition, elemental stoichiometry and therefore their value as manufacturing platforms (Shurin *et al.,* 2013).

1.6.1 Nutrients Availability

The availability of nutrients affects algae community composition and abundance of single species. The primary role of Phosphorous and Nitrogen has been widely studied (Kube et al., 2018), mainly because fertilisation with these inorganic elements has been recognised as a secure method to ensure dense algal population. However, future strategies should account for the avoidance of excessive nutrient loading so eluding downstream eutrophication, shift in the balance between tailored algal crops and invasive algal weeds and at the same time keeping optimal biomass growth and lipid content (Shurin *et al.,* 2013).

1.6.2 Light

Light wavelength and intensity are factors which directly affect both indoor and outdoor microalgal growth rates. In outdoor cultures, sunlight is the major source; conversely, in indoor cultures the biggest challenge is to overcome the high cost of artificial lighting. Microalgae absorb light of wavelengths in the range 400-700 nm for photosynthesis, with specific values varying for different species (Blair et al., 2014). Outdoor systems performance is lower than indoor ones and they also require large

land areas (Vieira Costa *et al.,* 2014). To maximise biomass productivity, light needs to be homogeneously distributed throughout the entire cultivation system, and avoid self-shading caused by high pond depth (Singh and Sharma, 2012) or biomass density (Sutherland et al., 2015).

1.6.3 Temperature

Temperature is an important factor influencing microalgal growth and hence target-product production. Regarding outdoor cultivations, temperature variations greatly depend on light exposure and seasonal changes. Appropriate temperature must be evaluated, as high values could lead to a decrease in biomass production caused by denaturation processes of proteins and enzymes (Yen *et al.,* 2013). Optimal temperature conditions are reported to be in the range 20-25°C for mesophilic algae species, up to 40°C for termophilic or down to 17°C for psychrophilic strains. (Ras et al., 2013).

1.6.4 Hydrodynamics of the Reactor

An adequate system mixing is necessary to provide high biomass concentration, allow medium circulation, keep the cells in suspension, avoid thermal stratification, optimize nutrients distribution, improve gas exchange and reduce shading and photo inhibition. (Vieira Costa *et al.,* 2014). Mechanical stirrers provide optimal efficiency both for mixing and gas transfer although causing significant hydrodynamic stress. On the other side, gas injection by impellers or airlift leads to low hydrodynamic stress, good gas transfer and acceptable mixing efficiency (Vieira Costa *et al.,* 2014).

1.6.5 Fixation of Carbon Dioxide

The fixation of CO_2 by algae has gained an increased attention due to the biomass production as it would allow reducing greenhouse gases emission and treatment of

industrial effluents. Generally, one kilogram of algal dry cell weight employs roughly 1.83 kg of carbon dioxide (Vieira Costa *et al.,* 2014). However, CO_2 concentration must not be too high as this could cause pH reduction and a consequently growth inhibition for some microalgae species (Wang *et al.,* 2012).

1.6.6 pH

The pH of the culture medium is an important parameter to be considered as it affects the characteristics of biochemical reaction of microalgae. It is crucial to keep culture pH in the optimal range (typically 7-9) because complete culture destruction may take place due to the disruption of cellular processes by extreme pH values. In any case, the control of pH needs to be integrated with the aeration system (Razzak *et al.,* 2015). In fact, in the case of cultivation with CO_2 addition, the concentration of this gas might be the predominant factor influencing the pH of the culture (Vieira Costa *et al.,* 2014). The optimal pH range for microalgae growth is species-dependent (Yen *et al.,* 2013).

1.6.7 Sterility of Cultivation

Contamination of cultures by different species of microalgae in open pond systems is controlled by effectively operating them as batch cultures and restarting the cultures at established intervals with new water and mono-algal inoculum. Contamination from insects, leaves and airborne materials must be controlled within acceptable limits as well. In open ponds, these contaminants are regularly removed by using a sized screen in the water flow (Yen *et al.,* 2013).

1.7 Scenedesmus spp. Cultivation in Open Pond Systems

The microalgae *Scenedesmus/Desmodesmus spp* are among the most commonly cultivated in open ponds all over the world (Benemann, 2013, Montemezzani, 2017). In any case, however, when planning the system design, several parameters must be

evaluated such as biology of the strain, cost of land and water, energy and nutrients requirements, local climatic conditions and target final product (Vieira Costa *et al.,* 2014).

1.7.1 *Scenedesmus* and *Desmodesmus* spp

Scenedesmus is a genus of the common non-motile freshwater green chlorophycean alga from the order *Sphaeropleales*. Their scientific classification is the following:

- Domain: *Eukaryota*
- Kingdom: *ViridiPlantae*
- Phylum: *Chlorophyta*
- Class: *Chlorophyceae*
- Order: *Sphaeropleales*
- Family: *Scenedesmaceae*
- Genus: *Scenedesmus/Desmodesms*

Scenedesmus and *Desmodesmus spp* can be found in freshwater bodies and even in the soil all over the world, as reported by Trainor in 1998. They are easily cultured and can both tolerate a wide range of environmental conditions, making them the ideal candidates to establish lab cultures (Lürling, 2003). More than a century ago, *Scenedesmus spp* were studied and reports of four celled colonies recorded, although placed under a different genus (*Achnantes*). It was only in early 1800s that Meyen first used the generic name *Scenedesmus* and therefore the genus is called *Scenedemus Meyen* in his honour (Lürling, 2003). Decades later, Chodat further subdivided in four the genus in four sub-genera*,* namely *Clathrodesmus*, *Desmodesmus*, *Euscenedesmus* and *Rhynchodesmus*. Fifty years after this classification, a new subdivision for the genus *Scenedesmus Meyen* was presented with the subgenera *Acutodesmus*,

Desmodesmus and *Scenedesmus*. Trainor and Hegewald then characterized the two groups as the non-spiny and the spiny group (Lürling, 2003). Only in recent years, there have been attempts to reassess the taxonomy of *Scenedesmus* by biochemical and physiological properties, which however failed. Therefore, molecular techniques such as nucleotide sequence analysis were later introduced to assist the reclassification of *Scenedesmus.* Sequence analysis of the 18S-rDNA gene clearly supported the designation of just two subgenera, *Desmodesmus* and *Scenedesmus.* Nevertheless, there is relatively low number of studies with *Desmodesmus* compared to those with Scenedesmus and this can be explained from investigators still being unaware of the division of the old genus *Scenedesmus* into the new genera *Scenedesmus* and *Desmodesmus* (Lürling, 2003).

1.7.2 Ultrastructure

Back in the 1990s, ultrastructural studies provided essential information on the architecture of *Scenedesmus* cell wall. Particularly, this alga is characterized by a three-layered cell wall made of cellulose, sporopollenin and both pectin and/or mucilage (Trainor, 1996). Some species have an outer cell membrane called “veil” connecting coenobia cells at their apices (Fig. 1-8) (Hegewald 1977).

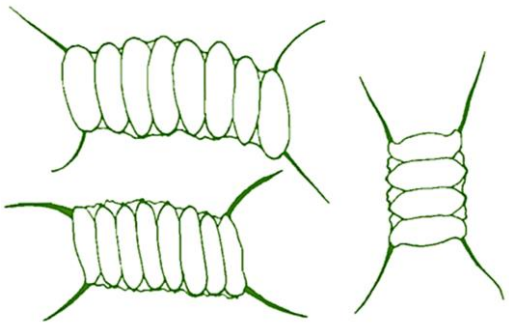


Fig.1-8 Representation of *Scenedesmus* colonies with a veil connecting cells (adapted from Trainor, 1996)

1.7.3. Morphology and Phenotypic Plasticity

It is widely reported in the literature that a single genotype can produce one or more alternative form of morphology in response to environmental conditions, a phenomenon called *phenotypic plasticity* (Lürling, 2003). Predation and competition are considered the primary selective forces responsible for the organization and structuring of communities. Of interest is the fact that zooplankton products of excretion can stimulate the formation of colonies, which has been interpreted as an induced defense (Hessen *et al.,* 1993, Lampert 1994, Lürling 1996).

1.7.4 Grazer-induced morphological changes in *Desmodesmus* and *Scenedesmus*

Members of the genus are characterized by the formation of *coenobia*. The *coenobium* is a special type of colony as it arises upon division of a single mother cell when the daughter cells stay connected by a common cell wall (Fig.1-9) (Bišová *et al.,* 2014).

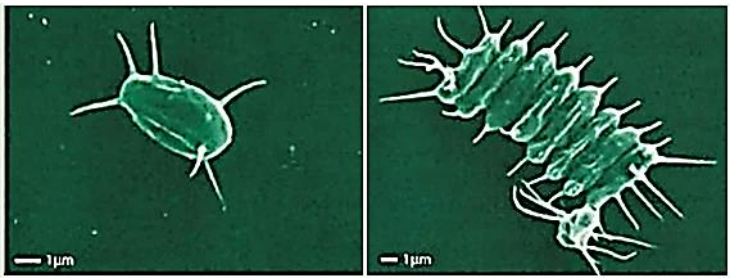


Fig.1-9 *Scenedesmus* unicell (left) and colony (right) (Hessen & Van Donk, 1993)

Desmodesmus and *Scenedesmus* show high variability not only in relation to the number of cells per colony but also in the size of the cells. Colony formation is a process reported as the algae defense against their grazers, *Daphnia spp* above all, along with other defensive induced characteristics like the formation of bristles and spines that may impede their ingestion. Other defensive features include an increase in cell wall thickness and production of mucilage (Lürling, 2003). Unicells and forms with bristles or spines are characterized by a greater resistance to sinking. Therefore,

unicells and small coenobia possessed better buoyancy than large coenobia allowing them to keep their position in the upper water layers with more favorable growth conditions. So, the cost to be paid by *Scenedesmus* and *Desmodesmus* to form protective colonies is, at least, an enhanced chance of sinking out of the euphotic zone (Lürling & Van Donk 2000). Among many factors, grazer chemical cues may begin the formation of eight-celled colonies that experience higher sinking rates (Lürling 2003). Important to mention is that the phenomenon of *Daphnia*-induced colony formation is not restricted to the genera *Desmodesmus* (Hessen & Van Donk 1993) and *Scenedesmus* (Lampert *et al.,* 1994, Lürling 1998). In fact, colonies can also form when *Coelastrum* (Lürling 1999, Van Donk *et al.,* 1999) or *Actinastrum* (Yasumoto *et al.,* 2000) are exposed to *Daphnia* chemical cues. Also, these induced defense mechanisms have been reported to be induced only by herbivorous zooplankton chemical cues and not by carnivorous zooplankton or fish, meaning that it is not about some more general animal excretory products, caused by the release of algal components *activated* only during the grazing process by digestive enzymes. (Lürling, 2003).

1.7.5 Reproduction

Scenedesmaceae usually reproduce asexually by the formation of autospores. Inside the parental cell wall, the mother cell experiences from 1 to 4 serial divisions into 2 to 16 daughter cells (Trainor, 1998). The daughter cells may be then released as a new colony varying in number of cells per colony by a simple unfolding. Less observed, *Scenedesmus* and *Desmodesmus* also undergo sexual reproduction (Trainor 1996).

1.8 Open Raceway Ponds as Natural ecosystems

To achieve algal productivity at a commercial scale as predicted by laboratory studies, it is necessary to deal with invasion by undesired organisms like predators and

competitors. The interactions between algae strains of industrial interest and these potential invading species basically follow the same dynamics of the extensively studied biotic interaction by ecologists worldwide. Lessons from Community Ecology, a sub field studying the organization and mechanisms of interacting species population on a local scale, would be particularly relevant to investigate the structure and dynamics of aquatic communities on an industrial scale, so providing an alternative strategy of microalgae cultivation to the simplistic elimination of organisms. In aquatic systems production often takes place in blooms, when microalgae rapidly reproduce in the water column. Aquatic communities are governed by a combination of two processes: the bottom-up and the top-down. As reported by Gliwicz in 2002, the bottom-up process is related to nutrient availability. Limiting nutrients are thought to determine the highest theoretical attainable biomass in aquatic systems. Also, light is a dominant limiting factor in large scale cultivation of microalgae, mainly because self-shading reduces the light penetration into the middle of dense cultures. At the same time, the produced biomass is also ruled by top-down processes, in the form of trophic cascades of predator-prey relationships (Fig. 1-10) (Kazamia *et al.,* 2012).

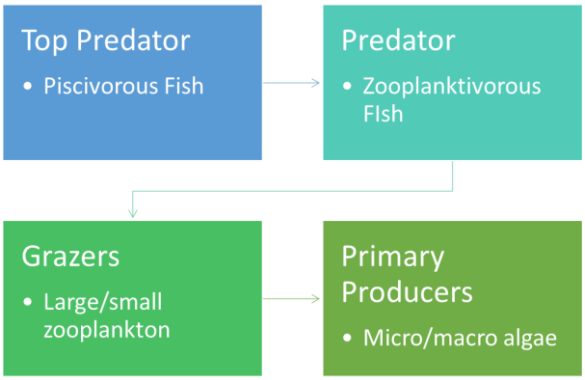


Fig.1-10 Schematic representation of trophic cascades (adapted from Kazamia *et al* 2012).

The combinations of the bottom-up and top-down concepts can facilitate the prediction of the dominant trophic levels in a determined aquatic community. However, other factors will also define the identity of the prevailing taxa inside each trophic level. In fact, if species share the same resources, niches or other limiting factors, they cannot co-exist on a long-term basis and the possible outcomes of species competition can be either the exclusion of one of the competitors or a rearrangement of the competitors' specialisation in relation to different resources or niches, which can then endorse a firm coexistence. This concept is known as the *competitive exclusion principle* (Kazamia *et al.,* 2012).

1.9 *Daphnia*: Physiology, Metabolism and Reproduction

Daphnia are planktonic crustaceans belonging to the Cladocera, whose bodies are enclosed by non-calcified double wall shell known as carapace, mainly composed by chitin (Fig. 1-11).

Fig. 1-11 *Daphnia* spp (courtesy of Dr D. Becker, University of Virginia)

Cladocera have up to 10 pairs of appendages, which are (from front to back): antennules, antennae (used for swimming), maxillae and mandibles followed by five limbs on the trunk. The limbs form an apparatus for feeding and respiration. At the end of the abdomen is a pair of claws. The body length of *Cladocera* ranges from less

than 0.5 mm to more than 6 mm. Compared to females, males have smaller size, larger antennules, modified post and first legs, which are armed with a hook used in clasping (Ebert *et al.,* 2005).

The genus *Daphnia* includes more than 100 known species of freshwater plankton organisms worldwide. All age classes are good swimmers and are mostly *pelagic*, which means they are mainly found in the open water bodies. They live as filter feeders, but some species may frequently be seen clinging to substrates such as water plants or even browsing over the bottom sediments of shallow ponds. The ecology of the genus *Daphnia* may be better known than the ecology of any other group of organisms (Ebert *et al.,* 2005). They feed on small, suspended particles in the water; although the feeding apparatus is so efficient that even bacteria can be collected, the food is usually made up of planktonic algae. Green algae are among the best food, and most laboratory experiments are performed with either *Scenedesmus* or *Chlamydomonas* species, both of which are easy to culture. *Daphnia* usually consume particles from around 1µm up to 50 µm, even though particles of 70 µm can be sometimes found in the gut content of large individuals (Ebert *et al.,* 2005).

Under ideal physicochemical conditions *Daphinidis* reproduce parthenogenetically producing clonal offsprings. However, a change in temperature or amount of available food may induce production of males with subsequent sexual reproduction and production of resting eggs. Apparently, parthenogenesis has evolved to let *Daphnia* taking advantage of good conditions as soon as they arise. Considering *Daphnia magna*, at a temperature of 20°C, it can reach sexual maturity in 6-8 days releasing its eggs into the brood pouch. The embryos then complete their development inside the brood chamber and hatch as free-swimming neonates at day 8-10. In the following 2-4 days, the mature females release a second brood of neonates with reproduction

peaking around the third brood (day 12-14) or fourth brood (day 14-17). Even under constant culturing conditions, brood size may vary due to parameters like water quality and/or crowding.

1.10 Importance of Species-Level Understanding & Concluding Remarks

Successful large-scale microalgae cultivation in open systems necessitates a good comprehension of species interactions, on the basis of which predictions can be made about how a community might develop and allowing the system optimisation towards a production aim. Algal cultivation could be improved by growing in a synthetic, engineered community with carefully selected players. It is possible to design such a community based on established ecological concepts and principles to keep a stable biomass production yield throughout the year (Kazamia et al., 2012). A synthetic community, having many of the usable compartments already occupied, so being opposed to natural ecological propensity for increased complexity, could be employed as a sustainable approach to industrial, commercial scale cultivation and harvesting of microalgae for low to medium value products.

Grazers have interactive mechanisms with algae which are gaining more and more attention, especially for what concerns their impact on nutrients uptake and community composition; these can be species-specific or determined by environmental factors or both (Lürling, 2003, Latta et al., 2009, Riessen et al., 2012, Eigemann *et al.,* 2013, O'Donnell 2013) and modulate trophic interactions (Pohnert *et al.,* 2007). However, there are still few studies in the literature taking into consideration the effects of grazing on algae communities in natural ecosystems and even less focusing on species identity of planktonic communities. Since microalgae are a wide group of different organisms, species-specific studies of algae are paramount

and difficult at the same time, as their heterogeneity implies that results generalization might not be always possible.

Many studies have suggested that planktonic food webs can be structured by chemically mediated interactions (Pohnert *et al.,* 2007) and the idea of exploiting natural cues from algae grazers and creating artificial ecosystems is gaining interest. However, it is still not clear how all the functional components of a synthetic complex can be well-established in an industrial relevant context, where it is essential to maintain a predictable and robust level of productivity. (Pandhal & Noirel*.,* 2014). Therefore, it is becoming necessary to find means to deal with, engineer and manipulate the interaction of microalgae with the other organisms in these artificially constructed ecosystems. The behavior of microalgae in natural environments, where comparable community dynamics subsist, is widely studied by freshwater ecologists so that it might be possible to make use of this deep knowledge of biotic interactions to promote better industrial practices (Kazamia *et al.,* 2012). This should represent a key point for future research, as an understanding of chemical cues structure and function mechanism will enable evaluating their potential impact on other organisms. Furthermore, synthetically produced chemical signals will allow performing large-scale tailored manipulations of interest. Future efforts should be directed towards understanding and taking advantage of these interactions, *natural or induced* (Shurin *et al.,* 2013) for the improvement of the whole algal biomass production process or at least its most critical steps such as harvesting.

REFERENCES – CHAPTER I

1. Acien FG, Fernandez JM, Magan JJ, Molina E. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnology Advances. 2012;30(6):1344-53.

2. Alam MA, Vandamme D, Chun W, Zhao X, Foubert I, Wang Z, et al. Bioflocculation as an innovative harvesting strategy for microalgae. Reviews in Environmental Science and Bio-Technology. 2016;15(4):573-83.

3. Bacellar Mendes LB, Vermelho AB. Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for Biofuels. 2013;6.

4. Benemann J. Microalgae for Biofuels and Animal Feeds. Energies. 2013;6(11):5869-86.

5. Bisova K, Zachleder V. Cell-cycle regulation in green algae dividing by multiple fission. Journal of Experimental Botany. 2014;65(10):2585-602.

6. Blair MF, Kokabian BG, Gude VG. Light and growth medium effect on Chlorella vulgaris biomass production. Abstracts of Papers of the American Chemical Society. 2014;247.

7. Bleeke F, Milas M, Winckelmann D, Klöck G. Optimization of freshwater microalgal biomass harvest using polymeric flocculants. International Aquatic Research. 2015;7(3):235-44.

8. Brennan L, Owende P. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable & Sustainable Energy Reviews. 2010;14(2):557-77.

9. Busi S, Karuganti S, Rajkumari J, Paramanandham P, Pattnaik S. Sludge settling and algal flocculating activity of extracellular polymeric substance (EPS) derived from bacillus cereus SK. Water and Environment Journal. 2017;31(1):97-104.

10. Davis ML. Water and Wastewater Engineering. USA: McGraw Hill Professional, 2010; 2010.

11. Demirbas A, Demirbas MF. Importance of algae oil as a source of biodiesel. Energy Conversion and Management. 2011;52(1):163-70.

12. Derjaguin BV. Untersuchungen über die Reibung und Adhäsion, IV. Colloid & Polymer Science. 1934;69(2):155-64.

13. Derjaguin B, Landau L. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged-particles in solutions of electrolytes. Progress in Surface Science. 1993;43(1-4):30-59.

14. Ebert D. Ecology, epidemiology, and evolution of parasitism in Daphnia. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information2005.

15. Eigemann F, Hilt S, Salka I, Grossart H-P. Bacterial community composition associated with freshwater algae: species specificity vs. dependency on environmental conditions and source community. Fems Microbiology Ecology. 2013;83(3):650-63.

16. Enzing C, Ploeg M, Barbosa M, Sijtsma L. Microalgae-based products for the food and feed sector: an outlook for Europe. Seville: IPTS Institute for Prospective Technological Studies, JRC; 2014.

17. Erkman S. Industrial ecology: an historical view. Journal of Cleaner Production. 1997;5(1-2):1-10.

18. Federico Ortiz-Marquez JC, Do Nascimento M, Zehr JP, Curatti L. Genetic engineering of multispecies microbial cell factories as an alternative for bioenergy production. Trends in Biotechnology. 2013;31(9):521-9.

19. Georgianna DR, Mayfield SP. Exploiting diversity and synthetic biology for the production of algal biofuels. Nature. 2012;488(7411):329-35.

20. Gliwicz ZM. On the different nature of top-down and bottom-up effects in pelagic food webs. Freshwater Biology. 2002;47(12):2296-312.

21. Ha K, Jang MH, Takamura N. Colony formation in planktonic algae induced by zooplankton culture media filtrate. Journal of freshwater ecology. 2004;19(1):9-16.

22. Habib MAB, Parvin M, Huntington TC, Hasan MR. A review on culture,

production and use of spirulina as food for humans and feeds for

domestic animals and fish. Rome: Food and agriculture organization of the united nations; 2008. Contract No.: 1034.

23. Hegewald E, Bock C, Krienitz L. A phylogenetic study on Scenedesmaceae with the description of a new species of Pectinodesmus and the new genera Verrucodesmus and Chodatodesmus (Chlorophyta, Chlorophyceae). Fottea. 2013;13(2):149-64.

24. Jakob G, Stephens E, Feller R, Oey M, Hankamer B, Ross IL. Triggered exocytosis of the protozoan Tetrahymena as a source of bioflocculation and a controllable

dewatering method for efficient harvest of microalgal cultures. Algal Research-Biomass Biofuels and Bioproducts. 2016; 13:148-58.

25. Jelinski LW, Graedel TE, Laudise RA, McCall DW, Patel CKN. Industrial Ecology - Concepts and Approaches. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(3):793-7.

26. Jena U, Hoekman SK. Advancements in Algal Biofuels Research – Recent Evaluation of Algal Biomass Production and Conversion Methods for Fuels and High Value Co-Products.: Frontiers Media SA; 2017.

27. Kazamia E, Aldridge DC, Smith AG. Synthetic ecology - A way forward for sustainable algal biofuel production? Journal of Biotechnology. 2012;162(1):163-9.

28. Kruyt HR. Colloid Science: Elsevier; Ex-library/volume I Irreversible Systems edition; 1952.

29. Kube M, Jefferson B, Fan L, Roddick F. The impact of wastewater characteristics, algal species selection and immobilisation on simultaneous nitrogen and phosphorus removal. 2018.

30. Lam MK, Lee KT. Microalgae biofuels: A critical review of issues, problems and the way forward. Biotechnology Advances. 2012;30(3):673-90.

31. Lam MK, Lee KT. Scale-Up and Commercialization of Algal Cultivation and Biofuel Production. Biofuels from Algae. 2014:261-86.

32. Lam MK, Lee KT, Mohamed AR. Current status and challenges on microalgae-based carbon capture. International Journal of Greenhouse Gas Control. 2012;

10:456-69.

33. Lammers PJ, Huesemann M, Boeing W, Anderson DB, Arnold RG, Bai XM, et al. Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Research-Biomass Biofuels and Bioproducts. 2017; 22:166-86.

34. Lampert W, Rothhaupt KO, Vonelert E. Chemical Induction of colony formation in a green-alga (Scenedesmus acutus) by grazers (Daphnia). Limnology and Oceanography. 1994;39(7):1543-50.

35. Latta IV, Leigh C, O'Donnell RP, Pfrender ME. Vertical distribution of Chlamydomonas changes in response to grazer and predator kairomones. Oikos. 2009;118(6):853-8.

36. Lee J, Cho D-H, Ramanan R, Kim B-H, Oh H-M, Kim H-S. Microalgae-associated bacteria play a key role in the flocculation of Chlorella vulgaris. Bioresource Technology. 2013; 131:195-201.

37. Lee S, Speight JG, Loyalka SK. Handbook of alternative fuel technologies: crc Press; 2014.

38. Liang Y, Hilal N, Langston P, Starov V. Interaction forces between colloidal particles in liquid: Theory and experiment. Advances in Colloid and Interface Science. 2007;134-35:151-66.

39. Lurling M. Effect of grazing-associated infochemicals on growth and morphological, development in Scenedesmus acutus (chlorophyceae). Journal of Phycology. 1998;34(4):578-86.

40. Lurling M. Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. Annales De Limnologie-International Journal of Limnology. 2003;39(2):85-101.

41. Lurling M. The effect of substances from different zooplankton species and fish on the induction of defensive morphology in the green alga Scenedesmus obliquus. Journal of Plankton Research. 2003;25(8):979-89.

42. Lürling M. The smell of water. Grazer-induced colony formation in Scenedesmus.: Agricultural University of Wageningen; 1999.

43. Lurling M, Van Donk E. Zooplankton-induced unicell-colony transformation in Scenedesmus acutus and its effect on growth of herbivore Daphnia. Oecologia (Berlin). 1996;108(3):432-7.

44. Manheim D, Nelson Y. Settling and Bioflocculation of Two Species of Algae Used in Wastewater Treatment and Algae Biomass Production. Environmental Progress & Sustainable Energy. 2013;32(4):946-54.

45. Marrone BL, Lacey RE, Anderson DB, Bonner J, Coons J, Dale, et al. Review of the harvesting and extraction program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Research. 2017.

46. Milledge JJ, Heaven S. A review of the harvesting of micro-algae for biofuel production. Reviews in Environmental Science and Bio-Technology. 2013;12(2):165-78.

47. Montemezzani V. Zooplankton Dynamics in Wastewater Treatment High Rate Algal Ponds and development of effective control methods: The University of

Waikato (NZ); 2017.

48. Montemezzani V, Duggan IC, Hogg ID, Craggs RJ. A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. Algal Research-Biomass Biofuels and Bioproducts. 2015; 11:211-26.

49. Moreno-Garcia L, Adjalle K, Barnabe S, Raghavan GSV. Microalgae biomass production for a biorefinery system: Recent advances and the way towards sustainability. Renewable & Sustainable Energy Reviews. 2017; 76:493-506.

50. Muradov N, Taha M, Miranda AF, Wrede D, Kadali K, Gujar A, et al. Fungal-assisted algal flocculation: application in wastewater treatment and biofuel production. Biotechnology for Biofuels. 2015;8(1):24.

51. Muylaert K, Bastiaens L, Vandamme D, Gouveia L. Harvesting of microalgae: Overview of process options and their strengths and drawbacks. WP; 2017.

52. O'Donnell DR, Fey SB, Cottingham KL. Nutrient availability influences kairomone-induced defenses in Scenedesmus acutus (Chlorophyceae). Journal of Plankton Research. 2013;35(1):191-200.

53. Pandhal J, Noirel J. Synthetic microbial ecosystems for biotechnology. Biotechnology Letters. 2014;36(6):1141-51.

54. Pohnert G, Steinke M, Tollrian R. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends in Ecology & Evolution. 2007;22(4):198-204.

55. Ras M, Steyer JP, Bernard O. Temperature effect on microalgae: a crucial factor

for outdoor production. Reviews in Environmental Science and Bio-Technology. 2013;12(2):153-64.

56. Rawat I, Kumar RR, Mutanda T, Bux F. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy. 2013; 103:444-67.

57. Razzak SA, Ilyas M, Ali SAM, Hossain MM. Effects of CO2 Concentration and pH on Mixotrophic Growth of Nannochloropsis oculata. Applied Biochemistry and Biotechnology. 2015;176(5):1290-302.

58. Rehman F, Medley GJD, Bandulasena H, Zimmerman WBJ. Fluidic oscillator-mediated microbubble generation to provide cost effective mass transfer and mixing efficiency to the wastewater treatment plants. Environmental Research. 2015; 137:32-9.

59. Renault F, Sancey B, Badot PM, Crini G. Chitosan for coagulation/flocculation processes - An eco-friendly approach. European Polymer Journal. 2009;45(5):1337-48.

60. Rizwan M, Lee JH, Gani R. Optimal design of microalgae-based biorefinery: Economics, opportunities and challenges. Applied Energy. 2015; 150:69-79.

61. Roberts BH. The application of industrial ecology principles and planning guidelines for the development of eco-industrial parks: an Australian case study. Journal of Cleaner Production. 2004;12(8-10):997-1010.

62. Roccuzzo S, Beckerman AP, Pandhal J. The use of natural infochemicals for sustainable and efficient harvesting of the microalgae Scenedesmus spp. for

biotechnology: insights from a meta-analysis. Biotechnology Letters. 2016;38(12):1983-90.

63. Rollie S, Mangold M, Sundmacher K. Designing biological systems: Systems Engineering meets Synthetic Biology. Chemical Engineering Science. 2012;69(1):1-29.

64. Ruiz J, Olivieri G, de Vree J, Bosma R, Willems P, Reith JH, et al. Towards industrial products from microalgae. Energy & Environmental Science. 2016;9(10):3036-43.

65. Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, et al. Biodiesel from algae: challenges and prospects. Current opinion in biotechnology. 2010;21(3):277-86.

66. Sen B, Alp MT, Sonmez F, Ali M, Kocer T, Canpolat O. Relationship of Algae to Water Pollution and Waste Water Treatment. In: Elshorbagy W, Chowdhury RK, editors. Water Treatment: InTech; 2013.

67. Sharma P, N S. Industrial and Biotechnological Applications of Algae: A Review. Journal of Advances in Plant Biology. 2017;1(1):1.

68. Show K-Y, Lee D-J, Chang J-S. Algal biomass dehydration. Bioresource Technology. 2013; 135:720-9.

69. Shurin JB, Abbott RL, Deal MS, Kwan GT, Litchman E, McBride RC, et al. Industrial-strength ecology: trade-offs and opportunities in algal biofuel production. Ecology Letters. 2013;16(11):1393-404.

70. Singh RN, Sharma S. Development of suitable photobioreactor for algae

production - A review. Renewable & Sustainable Energy Reviews. 2012;16(4):2347-53.

71. Snow AA, Smith VH. Genetically Engineered Algae for Biofuels: A Key Role for Ecologists. Bioscience. 2012;62(8):765-8.

72. Snow AA, Smith VH. Genetically Engineered Algae for Biofuels: A Key Role for Ecologists. Bioscience. 2012;62(8):765-8.

73. Sutherland DL, Craggs RJ. Utilising periphytic algae as nutrient removal systems for the treatment of diffuse nutrient pollution in waterways. Algal Research-Biomass Biofuels and Bioproducts. 2017; 25:496-506.

74. Sutherland DL, Howard-Williams C, Turnbull MH, Broady PA, Craggs RJ. Enhancing microalgal photosynthesis and productivity in wastewater treatment high rate algal ponds for biofuel production. Bioresource Technology. 2015; 184:222-9.

75. Tang YT, Rosenberg JN, Bohutskyi P, Yu G, Betenbaugh MJ, Wang F. Microalgae as a Feedstock for Biofuel Precursors and Value-Added Products: Green Fuels and Golden Opportunities. Bioresources. 2016;11(1).

76. Tibbs H. Industrial Ecology. An Environmental Agenda for Industry. Emerville, CA: Global Business Network; 1993.

77. Trainor FR. Reproduction in Scenedesmus. Algae. The Korean Journal of Phycology. 1996;11(2):183-201.

78. Trainor FR. Supplement to Nova Hedwigia; Biological aspects of Scenedesmus (Chlorophyceae) - phenotypic plasticity. [Supplement to Nova Hedwigia;

Biological aspects of Scenedesmus (Chlorophyceae) - phenotypic plasticity]. 1998;117: 367.

79. Uchida H, Yasumoto K, Nishigami A, Zweigenbaum JA, Kusumi T, Ooi T. Time-of-flight LC/MS identification and confirmation of a kairomone in Daphnia magna cultured medium. Bulletin of the Chemical Society of Japan. 2008;81(2):298-300.

80. Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy. 2010;2(1):012701.

81. Van Donk E. Defenses in phytoplankton against grazing induced by nutrient limitation, UV-B stress and infochemicals. Aquatic Ecology. 1997;31(1):53-8.

82. van Donk E, Lurling M, Lampert W. Consumer-induced changes in phytoplankton: Inducibility, costs, benefits, and the impact on grazers. The ecology and evolution of inducible defenses. 1999:89-103.

83. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

85. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology. 2013;31(4):233-9.

86. Verwey EJW. Theory of the stability of lyophobic colloids. Journal of Physical

and Colloid Chemistry. 1947;51(3):631-6.

87. Vieira Costa JA, de Morais MG. The role of biochemical engineering in the production of biofuels from microalgae. Bioresource Technology. 2011;102(1):2-9.

88. Vieira Costa JA, de Morais MG. An Open Pond System for Microalgal Cultivation. Biofuels from Algae. 2014:1-22.

89. von Elert E, Franck A. Colony formation in Scenedesmus: grazer-mediated release and chemical features of the infochemical. Journal of Plankton Research. 1999;21(4):789-804.

90. Wang B, Lan CQ, Horsman M. Closed photobioreactors for production of microalgal biomasses. Biotechnology Advances. 2012;30(4):904-12.

91. Yasumoto K, Nishigami A, Yasumoto M, Kasai F, Okada Y, Kusumi T, et al. Aliphatic sulfates released from Daphnia induce morphological defense of phytoplankton: isolation and synthesis of kairomones. Tetrahedron Letters. 2005;46(28):4765-7.

92. Yasumoto K, Nishigami A, Aoi H, Tsuchihashi C, Kasai F, Kusumi T, et al. Isolation of new aliphatic sulfates and sulfamate as the Daphnia kairomones inducing morphological change of a phytoplankton Scenedesmus gutwinskii. Chemical & Pharmaceutical Bulletin. 2008;56(1):133-6.

93. Yen H-W, Hu I-C, Chen C-Y, Chan J-S. Biofuels from Algae: Chapter 2. Design of Photobioreactors for Algal Cultivation.: Elsevier Inc. Chapters; 2013.

94. Zhu X, Yang J, Zhang X, Zhang L, Wang X, Huang Y, et al. Low temperature and

Daphnia-associated infochemicals promote colony formation of Scenedesmus obliquus and its harvesting. Biotechnology Letters. 2017;39(1):85-90.

95. Zimmermann W, Tesar V, inventors. Bubble generation for aeration and other purposes, 2013.

CHAPTER II

The role of Daphnia's Infochemicals on Scenedesmus spp. flocculation. Insights from a Meta-Analysis[1]

[1] This chapter is based on the paper published in Biotechnol Lett (2016) 38:1983–1990, DOI 10.1007/s10529-016-2192-2

2.1 INTRODUCTION

From the primordial soup ~3.5 billion years ago to today's water-bodies, all aquatic organisms have lived and live in an ocean of organic and inorganic chemicals which may play an important role in interactions among organisms (Lürling 1999). These can be either directly advantageous or disadvantageous or may induce physiological or behavioural responses. Many aquatic organisms use these information-conveying chemicals, referred to as infochemicals, to assess their risk of predation. Predator-induced defences are common among freshwater organisms like zooplankton and phytoplankton (Lürling 1999). however, very little is known about the role and the impact of infochemicals in the grazer-phytoplankton interactions.

In this thesis, the focus is on the grazing associated infochemicals produced by the zooplankton *Daphnia magna*, reported to induce a defence mechanism of colony formation in several microalgae species and strains to reduce their vulnerability against grazing. In a large-scale microalgal open cultivation system, infochemicals could be potentially used to induce defensive morphological and/or biochemical modifications in microalgae to promote colony formation and bio-flocculation. Controlled flocculation of microalgae through infochemicals is a technology giving grounds for expectations; however, these natural cues are likely to be highly species-specific. The underlying mechanism is still poorly understood and deserves further research because it may lead to a chemical-free method for flocculating microalgae.

In Science, it is not surprising to often find many studies basically considering the same question. Meta-analyses are defined as a systematic literature review supported by statistical methods aiming at the aggregation and comparison of the findings from various analogous studies (Viechtbauer 2010). Here, a systematic review and meta-

analysis of mostly ecology based studies was undertaken to assess the effects of infochemicals produced by the grazer *Daphnia magna* on colony formation and induction of bigger cells, in the form of Mean Particle Volume (MPV), of the microalgae *Scenedesmus spp*, determining the inter- and intra-specificity of their interactions. Parameters like phytoplankton strain, grazer's identity, feeding regime, density and incubation time were considered to determine the effect size of the "*Daphnia* Factor", so providing novel information about how much change in *Scenedesmus* particles size, expressed as either MPV or colony size, is evident across all studies and for subsets of studies. It is reported in the literature that a single *Scenedesmus* genotype can produce one or more alternative morphology form in response to environmental conditions (Lürling, 2003). Predation and competition are considered the primary selective forces responsible for the organization and structuring of communities. Of interest is the fact that *Daphnia* excretion products can stimulate the formation of colonies, interpreted as an induced defense (Hessen *et al.,* 1993, Lampert 1994, Lürling 1996). Members of the genus are characterized by the formation of *coenobia*, a special type of colony which arises upon division of a single mother cell when the daughter cells stay connected by a common cell wall (Bišová *et al.,* 2014). *Desmodesmus* and *Scenedesmus* species show high variability not only in relation to the number of cells per colony but also in the size of the cells.

2.2 WHY IS THIS IMPORTANT FROM AN ENGINEERING PERSPECTIVE?

Dewatering and harvesting of microalgae represent a primary bottleneck in the processing of biomass on an industrial scale, especially for low-medium value products such as biofuels. In fact, the highly dilute nature of the microalgal cultures leads to high operational costs during dewatering and harvesting therefore making algae less attractive, especially if compared to conventional agricultural biomass.

Harvesting by flocculation is generally considered a superior method to other procedures since it allows the treatment of high volumes of microalgae cultures and can be applied to a variety of species (Uduman *et al.,* 2010). Microalgae properties like large cell size may simplify this process as bigger cells would sink faster therefore enhancing their removal efficiency from the culturing medium. Also, particle size influences the structure of the formed flocs, their strength and therefore their resistance to breakage. The engineered use of naturally occurring *Daphnia* infochemicals would also induce the formation of grazing-resistant colonies. As the algae grow as unicellular, isolated cells when the predator *Daphnia* is absent, conversely, they experience a change in their morphology in the presence of the chemical warning cues, forming inedible bigger cells and colonies, more protected from grazing due to a size mismatch with its algae prey.

Several laboratory studies have been conducted to test these hypotheses but different methods of data reporting, the use of different experimental conditions or the lack of detailed information about both algae and grazers have made qualitative generalizations difficult and quantitative data is still missing. Here, several specific issues related to the industrial potential of *Daphnia* spp. infochemicals are addressed. First, natural cues may be highly species-specific and even strain/genotype specific. It is important to uncover any specificity as this could impact on strain selection for industrial biomanufacturing. Second, the effect size of grazer cues has never been estimated, which would allow standardized comparison among various grazers and importantly with the effects of chemical flocculants. Finally, the underlying mechanism of colony formation is still poorly understood; a systematic review facilitates insight into these mechanisms by synthesizing several metrics of colony size, including cell

number and overall floc size. A more comprehensive understanding of the mechanisms involved would lead to improved process control during algal cultivation.

This review cuts across several disciplines, data reporting methods, experimental conditions and importantly, the strain/genotype/species identity of grazer and algae. The present quantitative synthesis provides insight into the intra- and inter-specificity of algae (*S. obliquus*) – grazer (*Daphnia* spp) interactions associated with the production of colonies and a comparison between the effect size of biological cues and the effect size of commercially available chemicals.

2.3 METHODS

A research in Web of Science, StarPlus, Google Scholar, JStor and Mendeley databases was conducted with no constraint on publication year, using the following search term combinations: algae OR microalgae OR *Scenedemus* spp. OR *S. obliquus* OR Chlorophyceae OR Scenedesmaceae AND induced defences AND colony OR colony formation OR coenobia formation OR flocculation OR flocs OR aggregates OR morphology OR phenotypic plasticity OR mean particle volume AND grazers OR *Daphnia* OR Daphninids OR *Daphnia magna* OR Cladocerans OR chemical cues OR chemical signals OR infochemicals OR kairomones. This resulted in an initial set of 73 papers which were further screened, so that studies focusing on the impact of algae properties on grazers or those without replicates were excluded. When not readily available or clearly reported, data were extracted from graphs by use of WebPlotDigitizer, a web based tool to obtain quantitative data from plots, images and maps. When necessary, authors were asked to provide either raw data or relevant information (e.g. mean, standard deviation, sample size) when data could not be directly extracted from papers. Studies could not be included if estimates of variation and sample size were unavailable.

2.4 Effect Size Estimation

Effect sizes were estimated in the form of standardized mean difference, SMD, using the Cohen's d index. This is defined as "the unbiased standardized mean difference between an experimental group and its control" (Scheiner and Gurewitch, 2001) and it is calculated as the difference between the experimental and control mean-s divided by the pooled standard deviation, corrected if necessary by a factor accounting for small sample size (Equations. 1-1-1-3)

$$d_{ij} = \frac{\overline{x_{iJ}}^{E} - \overline{x_{iJ}}^{C}}{s_{ij}} J$$

Eq. 1-1. Cohen's d

Where:

- $\overline{x_{ij}}^{E}$ is the mean of the experimental group;
- $\overline{x_{ij}}^{C}$ is the mean of the control group;
- s_{ij} is the pooled standard deviation of the control and experimental groups;
- J is a corrective factor to account for bias due to small sample size.

$$J = 1 - \frac{3}{4\left(N_{ij}^{E} + N_{ij}^{C} - 2\right) - 1}$$

Eq. 1-2 Corrective Factor (Hedges and Olkin, 1985)

$$s_{ij} = \sqrt{\frac{\left(N_{ij}^{E} - 1\right)}{N_{ij}^{E} + N_{ij}^{C} - 2}}$$

Eq. 1-3 SD pooled

With:

- N_{ij}^{E} and N_{ij}^{C} as the size of the experimental and control groups, respectively;
- ${s_{ij}}^{E}$ and ${s_{ij}}^{C}$ as the standard deviations of the replicates in the experimental and control groups.

In general, the magnitude of the overall effect size is interpreted as small if the value of Cohen's d is 0.2, medium for d=0.5, large if d=0.8 and very large for d ≥1 (Riessen, 1999). Also, it can be assessed that there are significant differences between control and experimental groups if the 95% Confidence Intervals (CI) around d do not overlap zero (Sheiner & Gurevitch, 1993). We conducted a random-effects meta-analysis using R (R Core Team, 2015) and the package *metafor* (Viechtbauer, 2010). In every study, SMD was calculated from the difference between a treatment with infochemicals or flocculant and a control, represented by algae only.

2.5 Hypotheses

The Grazer Specificity hypothesis that species identity of cladoceran grazers will induce differential responses in the same algae species/strain was tested, followed by the Algae Specificity hypothesis, where for a single species of grazer (*D. magna*), whether different strains of the same algae species respond differently to the same grazer infochemicals was investigated. The hypotheses that a) grazer feeding duration, b) incubation time of grazer and algae together and c) the grazer density used to produce the infochemicals, affected algae colony formation were also examined. Finally, to explore the potentialities of grazers' cues in algal biotechnology, an investigation on whether *Daphnia* infochemicals can induce comparable responses in Scenedesmus to two chemical surfactants with a similar chemical structure to some of those proposed for *Daphnia's* infochemicals (FFD-6, a surfactant solution made of

55% water and 45% mono- and didodecyl disulphanated diphenyloxide, sodium salt, and sodium dodecylsulfate (Lürling and Beekman, 2002, Lürling, 2006)) was accomplished.

2.6 Results

After screening for standard meta-analytic criteria (sample size, mean and standard deviations reported), the final data set comprised nine studies and 85 trials (Lampert et al., 1994, Lürling & van Donk, 1996, Lürling & van Donk, 1997, Lürling, 1998, Lürling, 1999, Lürling, 2000, Lürling & Beekman, 2002, Lürling, 2003, Lürling, 2006). Studies document effects of several cladoceran grazers: *Daphnia pulicaria, D. pulex, D. magna, D. cucullata, D. galeata, D. galeata x hyalina* and *Ceriodaphnia reticulata*. The *Scenedesmus obliquus* strains represented were UTEX 78, UTEX 1450, UTEX 2630, SAG 276/3A, SAG 276/1 and NIVA CHL6.

2.6.1 Grazer Specificity

Five studies provided 46 trials to compare the response of the mean particle volume (MPV) of *Scenedesmus obliquus*, strain SAG 276/3A to infochemicals produced by seven grazer species. MPV was measured using a coulter counter and uses electrical impedance to measure the volume of particles as they pass individually through an aperture of defined size. In all studies, data were obtained by using filtered (0.1 - 0.2 µm) water sourced from tanks where individuals could graze on algae for 24 h. Filtrate water was added in all studies at concentrations between 4% and 10% v/v. Chemical cues in water from grazing *Daphnia* spp were found to increase the MPV of *Scenedesmus obliquus*, strain SAG 276/3A ($Q_{(df=45)} = 284.7702$), $p < 0.001$). Grazer specificity was also detected (Fig.2-1; Table 2-1); specifically, *D. pulicaria* produced the strongest effect, one that was double the average effect size of all other grazers. *D. magna, D. galeata, D. galeata x hyalin*a and *C. reticulata* all induced colony formation

at a moderate effect size. The effects of *D. pulex* and *D. cucullata* could not be distinguished from the control.

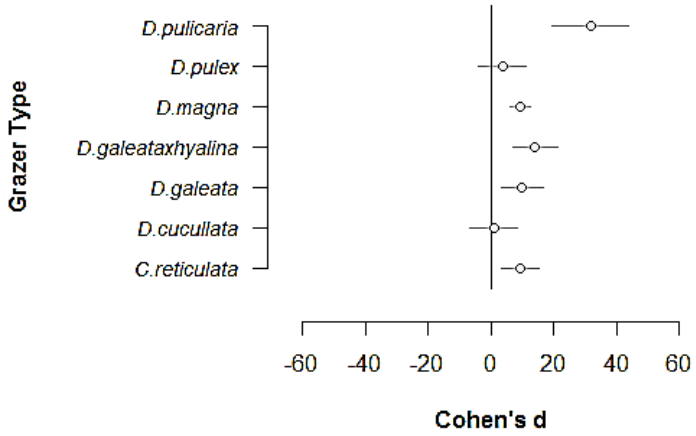


Fig.2-1 The effect of grazer (*Daphnia* spp) identity on mean particle volume (MPV) of *S. obliquus*, strain SAG276/3A. Data are mean ± 95CI of Cohen's d, estimated from a random effects meta-analytic model of the effect of grazing after two days of exposure.

Table 2-1 Results of a random effects meta-analytic model of the effect of grazing. n- sample size, CI – confidence interval

Type of Grazer	Effect Size	Lower 95% CI	Upper 95% CI	n
D. pulicaria	31.75	19.52	43.99	24
D. pulex	3.58	-4.01	11.18	18
D. magna	9.32	6.04	12.60	194
D. galeata x hyalina	14.08	6.92	21.23	30
D. galeata	9.92	3.26	16.57	30
D. cucullata	0.88	6.60	8.37	24
C. reticulata	9.31	3.42	15.21	36

2.6.2 Algae Strain Specificity

Five studies providing 29 trials allowed the comparison of the response of various strains of *S. obliquus* to infochemicals produced by *Daphnia magna*. Main findings consisted in filtered *D. magna* water inducing larger MPV overall (Q (df = 28)

=189.9879, p < .0001). There were no significant differences among the strains (Fig. 2-2; omnibus p =0.9424).

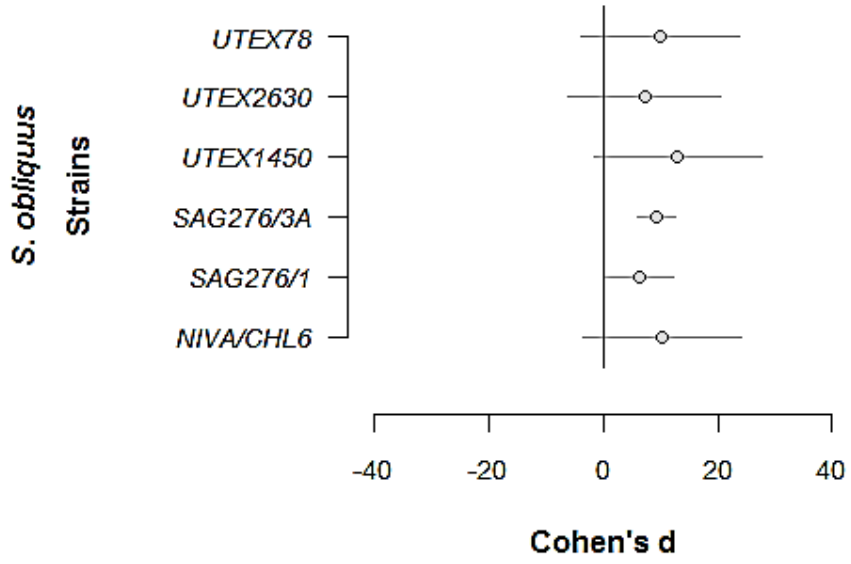


Fig. 2-2 The change in mean particle volume (MPV) of six strains of *S. obliquus* exposed to filtered water from *D. magna* cultures. Data are mean ± 95CI of Cohen's d, estimated from a random effects meta-analytic model of the effect of grazing.

2.6.3 Starvation, Duration of Incubation and Density of Grazers

Data for comparing the effects on algal MPV where *Daphnia magna* grazers were fed or starved were sourced from two studies with six trials with infochemicals from starved animals and seven studies with 50 trials for fed individuals. Time of exposure and grazers' density effects were evaluated with data from seven studies and 56 trials. Water filtered from fed animals was found to increase MPV (d=12.5655, CI (8.5666;16.5645), but the effect of starved animals was highly variable (n = 6 trials) and could not be distinguished from zero (Fig.2-3, d= 3.5318, CI (-1.6293; 8.6929).

For the case of *D. magna,* no differences were associated with one, two or three days of exposure to infochemicals (p = 0.8646) as well as no differences due to culture densities used to produce the infochemicals (p = 0.7374).

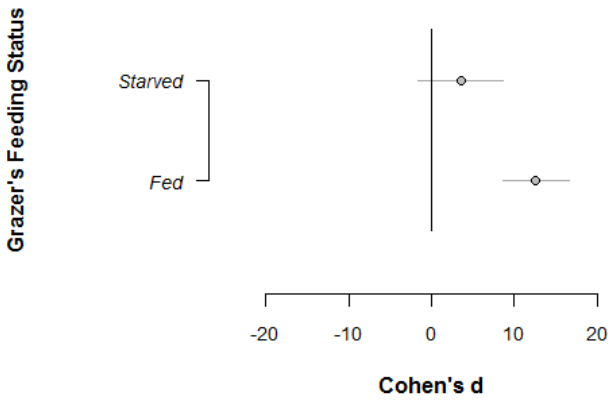


Fig.2-3 The effect of *Daphnia magna* food intake or starvation on mean particle volume of *S. obliquus*, strain SAG 276/3A. Data are mean ± 95CI of Cohen's d, estimated from a random effects meta-analytic model of the effect of grazing feeding status.

2.6.4 Effect Size Comparison

A strong concentration dependent effect of both grazer (Fig. 2-4/A) and surfactants (Fig. 2-4/B) was uncovered. *D. pulicaria* produces double the effect size of the other grazers, and does so at dramatically lower densities (5-20 animals per liter). Furthermore, comparing the effect sizes of *D. pulicaria* with surfactants FFD-6 and SDS shows that grazer infochemicals can rival or even outperform induced changes in MPV caused by the commercially available surfactants. It is important to emphasize that the grazer data is for 2-day trials thus several grazer species produce effect sizes of similar or much greater magnitude (e.g. *D. pulicaria*) in half the incubation time of FFD-6.

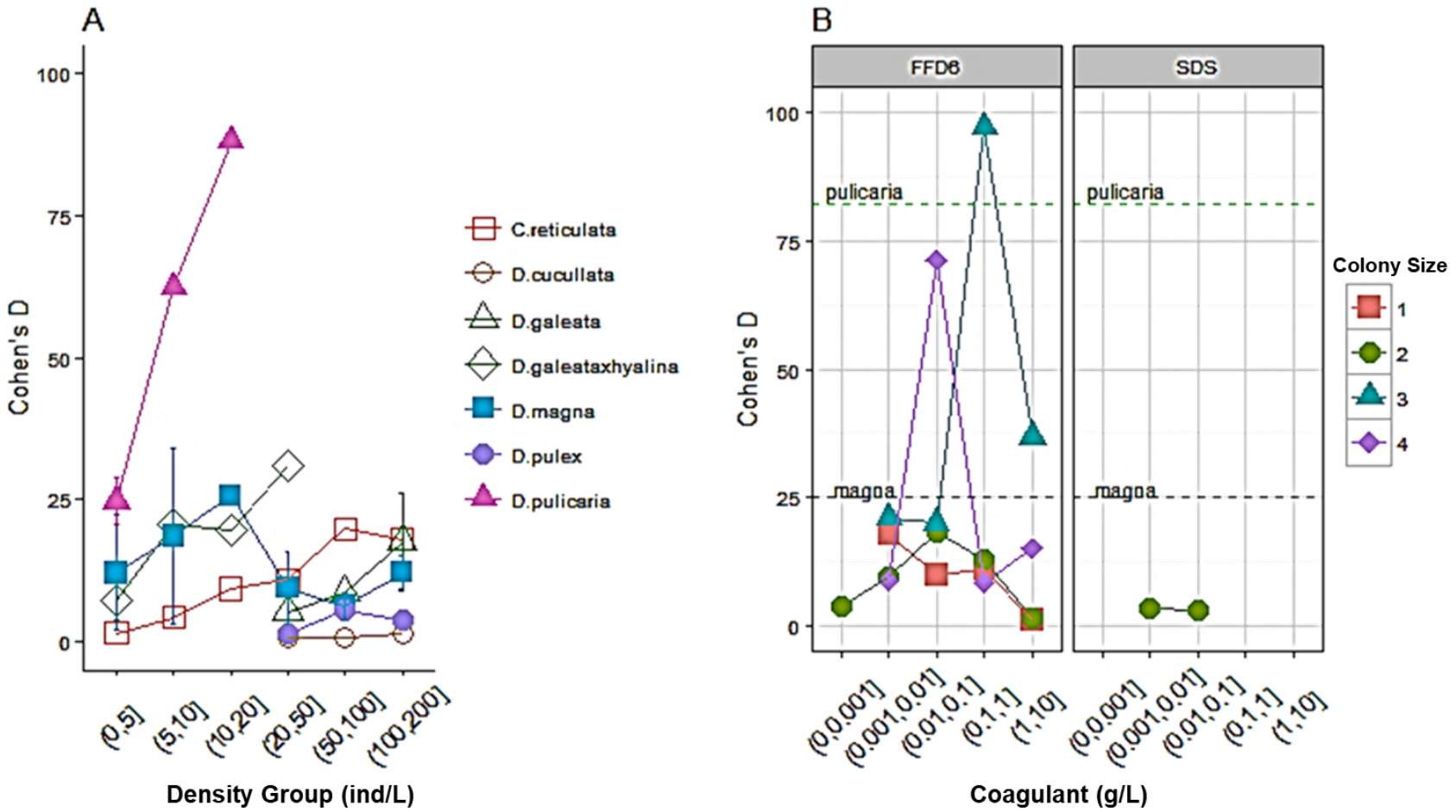


Fig.2-4 Comparison and contrast of mean effect sizes of S. obliquus mean particle volume induced variation, as affected by grazers culture density (Panel A) and surfactants concentration levels (Panel B).

2.7 Discussion

The main objective of this chapter was to quantitatively evaluate the potential for cladoceran grazer infochemicals to induce colony formation, a phenomenon which might be exploited for microalgae flocculation and hence, biomass harvesting for biotechnology. Whether grazer species identity altered colony formation (grazer specificity) and whether different *S. obliquus* strains responded differentially to a common grazer (algae specificity) were specifically addressed. It was important to understand specificity of colony formation as it entails an additional trait for selecting algal strains for large scale cultivation in biomanufacturing (addition to productivity, growth rates, resistance to diseases etc.), ultimately impacting on downstream processing. An evaluation, via standardized effect sizes, whether grazer infochemicals generated effects at all like commercially available chemical surfactants, FFD-6 and SDS was done. These findings suggest that cladoceran infochemicals show substantial promise: a significant effect of grazer identity, an effect size similar, or even higher under certain conditions, than commercial surfactants and no differences related to algae strains differentiation was found. However, data available were surprisingly constrained. Out of >70 possible papers, only nine studies with 85 trials offered data in a format to be included in the meta-analysis. Such low reporting rates of variation (e.g. standard deviation) and of sample sizes clearly hinders the ability to identify what appears to be a potentially positive use of infochemicals in industry.

2.7.1 Specificity and *D. pulicaria*

One of the most surprising outcomes associated with the current assessment of grazer specificity was that the most commonly used species here, *D. magna*, reported in more than 50% of the published papers, is relatively poor at inducing cell volume change. Instead, the relatively little studied *D. pulicaria*, appears able to produce

infochemicals with the largest effect size, doubling the average of all the other grazers under study during the same incubation time (48h; Fig. 2-4A). To be highlighted is the capacity of *D. pulicaria* to induce changes in particle volume which was not only higher than all other grazers, but generated these responses at very low culture densities, suggesting high promise. It is to be noticed however the small amount of data, requiring much more research. In addition to the standout effects of *D. pulicaria*, several other species "outperformed" the commonly cultured *D. magna. D. galeata x hyalina* also shows promise with a steadily rising effect on MPV that may continue to escalate at higher culture density (Fig. 2-4 A).

2.8 Infochemicals as Novel Algal Flocculants

The advantage of using natural infochemicals over traditional coagulants include potentially lower costs, a more sustainable and environmentally friendly production process and reduced contamination of the growth media and feedstock. Although a comparison to traditional coagulants was not a motive in this meta-analysis, it was possible to calculate the standardization of effect size and assess whether natural infochemicals can induce changes similar to that of commercially available surfactants. Figs.2-4 A, B strongly suggest that infochemicals from more than one species have the potential to generate effects on the same scale as FFD-6 and well beyond SDS.

2.9 Conclusions

This meta-analysis suggests the next steps from both an engineering and biotechnology perspective: designing methods to provide infochemicals rich water for harvesting algal biomass that may be centered on recirculation of Daphnia cues medium. A potential biochemical agenda of identifying the chemical composition and species specificity of the infochemicals and ultimately their capacity for synthesis within an integrated system is highlighted. This is the first quantitative assessment of

the importance of microalgae-grazers species-specific interactions and findings disclose the potential for developing an integrated bio-flocculation system based on natural infochemicals in open raceway ponds.

REFERENCES – CHAPTER II

1. R: A language and environment for statistical computing. Vienna, Austria: Foundation for Statistical Computing; 2015.

2. Bacellar Mendes LB, Vermelho AB. Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for Biofuels. 2013;6(1):152.

3. Bacellar Mendes LB, Vermelho AB. Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for biofuels. 2013;6(1):152-.

4. Farooq W, Moon M, Ryu B-g, Suh WI, Shrivastav A, Park MS, et al. Effect of harvesting methods on the reusability of water for cultivation of Chlorella vulgaris, its lipid productivity and biodiesel quality. Algal Research-Biomass Biofuels and Bioproducts. 2015; 8:1-7.

5. Gong YM, Jiang ML. Biodiesel production with microalgae as feedstock: from strains to biodiesel. Biotechnology Letters. 2011;33(7):1269-84.

6. Hedges LV, Olkin I. Statistical Methods for Meta-Analysis. New York: Academic Press.

7. Hessen DO, Vandonk E. Morphological changes in Scenedesmus induced by substances released from Daphnia. Archiv Fur Hydrobiologie. 1993;127(2):129-40.

8. Lampert W, Rothhaupt KO, Vonelert E. Chemical Induction of colony formation in a green-alga (Scenedesmus acutus) by grazers (Daphnia). Limnology and Oceanography. 1994;39(7):1543-50.

9. Lürling M, Van Donk E. Zooplankton-induced unicell-colony transformation in

Scenedesmus acutus and its effect on growth of herbivore Daphnia. Oecologia (Berlin). 1996;108(3):432-7.

10. Lurling M. Effect of grazing-associated infochemicals on growth and morphological, development in Scenedesmus acutus (chlorophyceae). Journal of Phycology. 1998;34(4):578-86.

11. Lurling M. Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. Annales De Limnologie-International Journal of Limnology. 2003;39(2):85-101.

12. Lurling M. The effect of substances from different zooplankton species and fish on the induction of defensive morphology in the green alga Scenedesmus obliquus. Journal of Plankton Research. 2003;25(8):979-89.

13. Lurling M. Effects of a surfactant (FFD-6) on Scenedesmus morphology and growth under different nutrient conditions. Chemosphere. 2006;62(8):1351-8.

14. Lurling M, Beekman W. Extractable substances (anionic surfactants) from membrane filters induce morphological changes in the green alga Scenedesmus obliquus (Chlorophyceae). Environmental Toxicology and Chemistry. 2002;21(6):1213-8.

15. Lurling M, Van Donk E. Morphological changes in Scenedesmus induced by infochemicals released in situ from zooplankton grazers. Limnology and Oceanography. 1997;42(4):783-8.

16. Lürling M. The smell of water. Grazer-induced colony formation in Scenedesmus.: Agricultural University of Wageningen; 1999.

17. Lürling M. Grazer-induced coenobial formation in clonal cultures of Scenedesmus obliquus (Chlorococcales, Chlorophyceae). Journal of Plankton Research. 1999a; 23:19-23.

18. Lürling M, Van Donk E. Grazer-induced colony formation in Scenedesmus: are there costs to being colonial? Oikos. 2000;88(1):111-8.

19. Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: A review. Renewable & Sustainable Energy Reviews. 2010;14(1):217-32.

20. Mayeli SM, Nandini S, Sarma SSS. The efficacy of Scenedesmus morphology as a defense mechanism against grazing by selected species of rotifers and cladocerans. Aquatic Ecology. 2004;38(4):515-24.

21. Montemezzani V, Duggan IC, Hogg ID, Craggs RJ. A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. Algal Research-Biomass Biofuels and Bioproducts. 2015; 11:211-26.

22. Oh HM, Lee SJ, Park MH, Kim HS, Kim HC, Yoon JH, et al. Harvesting of Chlorella vulgaris using a bioflocculant from Paenibacillus sp AM49. Biotechnology Letters. 2001;23(15):1229-34.

23. Pohnert G, Steinke M, Tollrian R. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends in Ecology & Evolution. 2007;22(4):198-204.

24. Riessen HP. Predator-induced life history shifts in Daphnia: a synthesis of

studies using meta-analysis. Canadian Journal of Fisheries and Aquatic Sciences. 1999;56(12):2487-94.

25. Scheiner SM, Gurevitch J. Design and analysis of ecological experiments.: Oxford University Press; 2001.

26. Shelef G, Sukenik A, Green M. Microalgae harvesting and processing: a literature review. Technion Research and Development Foundation Ltd., Haifa (Israel); 1984. Contract No.: No. SERI/STR-231-2396.

27. Shen Y, Fan Z, Chen C, Xu X. An auto-flocculation strategy for Chlorella vulgaris. Biotechnology Letters. 2015;37(1):75-80.

28. R. T, Harvell CD. The ecology and evolution of inducible defenses.: Princeton University Press; 1999.

29. Tollrian R, Harvell CD. The ecology and evolution of inducible defenses. The ecology and evolution of inducible defenses. 1999

30. Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy. 2010;2(1):012701.

31. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

32. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology.

2013;31(4):233-9.

33. Viechtbauer W. Conducting Meta-Analyses in R with the metafor Package. Journal of Statistical Software. 2010;36(3):1-48.

34. Wu X, Zhang J, Qin B, Cui G, Yang Z. Grazer density-dependent response of induced colony formation of Scenedesmus obliquus to grazing-associated infochemicals. Biochemical Systematics and Ecology. 2013; 50:286-92.

35. Zhu X, Nan H, Chen Q, Wu Z, Wu X, Huang Y, et al. Potential grazing intensity directly determines the extent of grazer-induced colony formation in Scenedesmus obliquus. Biochemical Systematics and Ecology. 2015; 61:271-7.

CHAPTER III

An Experimental Evaluation of Infochemicals Effects on Scenedesmus subspicatus

3.1 INTRODUCTION

Despite a great variety of available microalgae harvesting methods, flocculation is considered one of the most promising economic approaches for pre-concentrating very large amounts of biomass, ultimately facilitating cell harvesting and reducing processing costs (Barros *et al.,* 2015). Flocculation leads to aggregation of dispersed cells, increasing particles sizes and improving rates of sedimentation or flotation. Although flocculation can be achieved by several well-established methodologies, the most common relies on the use of metal salts like ferric chloride, which induce flocculation by means of charge neutralization. However, this results in an accumulation of metals in the system, which contaminates the biomass and medium, interfering with the final use of biomass itself or impeding recycling of the medium in cultivation vessels. Polymers like chitosan are also used, representing a safer but more expensive alternative to metal salts. Just as expensive are physical methods like the use of electromagnetic pulses. These avoid biomass contamination but are difficult to be applied on large scale (Vandamme *et al.,* 2013). Altering process parameters such as temperature or pH can also induce flocculation. However, these processes are difficult to control and can have undesirable consequences on cell composition (Benemann & Oswald, 1996).

Bio-flocculation is another approach that can be successfully exploited to harvest microalgae biomass at large scale. Methods include addition of bacteria (Van Den Hende et al.,2011, Busi et al., 2017) or auto-flocculating algae (Lananan et al., 2016, Ummalyma et al., 2016) which can be however species-specific, slow and unreliable (Milledge and Heaven, 2013). Flocculation of microalgae could be also achieved through genetic engineering of the strain of interest to gain flocculating properties (Gomaa et al., 2016). In this case however, it is important to highlight that for

industrial, large scale production it is almost impossible to work under containment with the consequent risks of escape of genetically modified algae into the environment (Wijffels et al., 2013).

In this thesis, an investigation of the use of naturally occurring infochemicals produced by grazers of algae as a potentially sustainable bio-flocculation method is presented. Infochemicals are substances released by zooplankton grazers that induce algae species specific defensive mechanisms against predation. Aspects of these defences are well studied in the ecological literature and include formation of colonies and bio-flocculation. However, they have not been evaluated specifically in the context of harvesting for biotechnology applications. Harvesting microalgae with infochemicals would avoid the use of contaminating substances like metals, enable recycling of the cultivation medium and not require expensive options such as altering cultivation conditions. The most studied system of these defensive responses centre on the microalgae *Scenedesmus* spp. and the grazers *Daphnia* spp. (Hessen and van Donk 1993, Lampert *et al.,* 1994, Lürling 1999, Lürling 1999a, Lürling 2003, van Holthoon *et al.,* 2003, Pohnert *et al.,* 2007, O'Donnell *et al.,* 2013, Wu *et al.,* 2013, Zhu *et al.,* 2015). A meta-analysis, summarising much of this work from a biotechnology perspective, highlights grazer-specific levels of colony formation, and suggests that a distinction between colony formation and aggregation-based mechanisms is necessary (Roccuzzo *et al.,* 2016). Colony formation is typically interpreted as an altered cell division process leading to multicellular entities (Bišová et al., 2014). Aggregation defines a process of adhesion among existing dispersed cells (Li and Guo, 2016).

The focus is on deciphering the mechanisms inducing bio-flocculation through colony formation and/or aggregation, knowledge that is important to successfully incorporate this natural phenomenon into engineered operations such as microalgae cultivation

systems in open raceway ponds. Results are specifically detailed from experimental exposure of *Scenedesmus subspicatus* to *Daphnia magna* infochemicals, reporting on changes growth rates and cell number, colony formation and adhesion of existing cells. These data are analysed at three stages of algae growth and at five concentrations of infochemicals. Theory suggests several assays can be used to distinguish between colony formation/cell division processes and aggregation/adhesion processes. First, assays of growth rates and cell number can be used to first infer whether during the flocculation process cell numbers increase as a function of accelerated growth rates, suggesting an effect on cell division processes. Second, colony formation in *S subspictus* is defined as an altered cell division process producing objects called *coenobia*, which occur in powers of 2 cells (ie. 2, 4, 8, 16 cell colonies) (Zachleder et al., 2011). The abundance of these can be monitored as well. Third, the morphology of flocs themselves can be assayed using image analysis to identify their size and structure and therefore give indications on the flocculation mechanism (Li et al., 2006). These parameters are also regarded as fundamental for the operation of industrial processes (Jarvis et al., 2005), affecting the efficiencies of particles separation (Li et al., 2006). Fourth, assessment of flocculation efficiency (e.g. settling rates) can be used in a dose response experiment to evaluate its performance across various conditions and to also investigate on the mechanism involved; in fact, under a model of cell surface charge neutralisation (see Chapter I), a quadratic flocculation rate with increasing infochemicals dose is expected, with efficiency lowest at high and low doses (Billuri et al., 2015, Guo et al., 2015). In contrast, under a cell-cell adhesion process, flocculation efficiency is hypothesised to increase linearly with increasing infochemicals dose (Fig. 3-1)

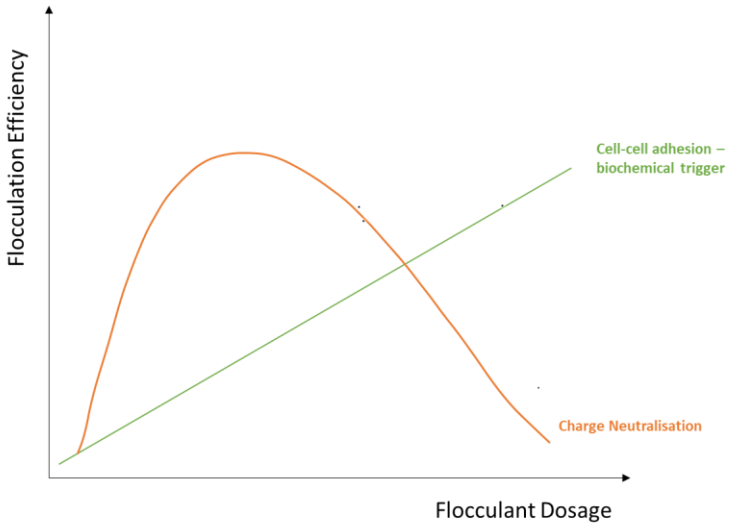


Figure 3-1 Schematic representation of proposed flocculation mechanisms: charge neutralisation (orange) and cell-cell adhesion (green)

Finally, FT-IR analyses can reveal potential changes induced in algae cell surface characteristics such as concentration and reactivity of functional groups on the cell wall (Alonso-Simón et al., 2011). In bacteria, FT-IR investigations have been successfully applied to reveal different cell surface properties and to distinguish between freely suspended cells (planktonic) and cells living in biofilms (Karunakaran and Biggs, 2011, Mukherjee et al., 2012). FT-IR studies could also reveal whether the flocculation process occurs through bridging between cells, which would be in fact reflected in additional absorption peaks in spectrum (Liu et al., 2015).

Here, all of these methods were combined in a systematic assessment of flocculation potential in *S. subpsicatus* generated by chemical cues from the microcrustacean *Daphnia magna*. The overall objective is to evaluate the feasibility and efficiency of this bio-flocculation approach through the assessment of key parameters like initial algal concentration and culture cultivation stage, flocculant dosage, flocs size and cell surface characteristics.

3.2 Methods

3.2.1 Algae and Daphnia Culture Conditions

S. subspicatus NIVA-CHL 97 was obtained from the Norwegian Institute for Water Research (NIVA-CCA) and maintained in the lab in Ebert's medium (Table 3-1). The alga was cultured in 250 mL Erlenmeyer flasks at 20 ±1°C on a shaking table at 120 rpm and continuously illuminated from above with a light at 259 µmol/m^2·s. The grazer *Daphnia magna* used to produce the infochemicals was reared in a temperature controlled room at 20±1°C in a 16:8 light-dark cycle, cultured in one L jars with artificial pond water (ASTM, 1980) and fed daily with *S. subspicatus* at a concentration of 2·10^5 cells/mL.

Table 3-1 *Ebert' medium composition*

Chemical	**Concentration [g/L]**
$CaCl_2$	0.0368
$MgSO_4\ 7H_2O$	0.037
$NaHCO_3$	0.0126
$K_2HPO_4\ 3H_2O$	0.0114
$NaNO_3$	0.085
$Na_2SiO_3\ 5\ H_2O$	0.0212
$NaEDTA$	0.00436
$FeCl_3\ 6\ H_2O$	0.00315
$CuSO_4\ 5\ H_2O$	0.00001
$ZnSO_4\ 7\ H_2O$	0.000022
$CoCl_2\ 6\ H_2O$	0.00001
$MnCl_2\ 4\ H_2O$	0.00018
$Na_2MoO_4\ 2\ H_2O$	0.000006
H_3BO_3	0.001

3.2.2 Infochemicals Production and Experimental Design

To produce the infochemicals, *D. magna* were incubated at a density of 100 ind/L with *S. subspicatus* as food. After 24 h, animals were removed and the culture filtered through a 0.2 µm cellulose acetate filter (Sartorius Stedim Biotech Gmbh, Germany) to obtain the *Daphnia* test water (DW). Five mL of exponentially growing *S. subspicatus*

(~10^6 cells/mL) was transferred to 250 mL Erlenmeyer flasks containing 150 mL of autoclaved Ebert's medium and either five mL of additional culture medium or five mL of DW. Batch cultures were incubated at 20±1°C on a shaking table at 120 rpm, continuously illuminated from above by light tubes at 259 µmol/m^2·s and randomly rearranged daily. DW was applied at four levels, defined by full concentration and three serial 10-fold dilutions (*DW 1:10, DW 1:100, DW 1:1000*). These concentrations defined the dose – response axis to assess effects of grazer cues on *S. subspicatus*. Each treatment was replicated four times during each of the three different growth stages: early exponential (five days), late exponential (ten days) and stationary phase (15 days). At each stage, algae were exposed to infochemicals for 20 h.

3.2.3 Composition and Growth

Aliquots of algae (one mL) were taken every other day and fixed in Lugol's dilute solution. Growth rates and composition (unicells and *coenobia*) were determined by cell counting, using a haemocytometer (Neubauer Improved Superior, Germany) under a microscope (Kyowa, Medilux-12) and reported as (cells/mL vs. day) and percentage distributions of unicells, 2-, 3-, 4-, 8- celled colonies, respectively. Growth curves were fitted by non-linear regression specifying a Michaelis-Menten model for counts between day 1 and 16. The Michaelis-Menten model has an asymptote (V_m), representing the maximum growth rate at saturating substrate and half-saturation value (k) representing the day at which growth is ½ max. Vm and k were estimated for each replicate and these estimates used to statistically compare treatments using ANOVA and a post-hoc Tukey test. The cell number and composition of flocs was determined by mechanical disaggregation, followed by counting of the constitutive cells (unicells and/or colonies) using the haemocytometer and microscope described above.

3.2.4 Morphology of Flocs

For all DW treatments and at each growth stage, flocs were collected from the bottom of the flasks and carefully placed on a glass slide using a wide mouth pipette to avoid physical damage and covered with a glass cover sheet. Images were captured using a microscope (Leitz Wezler, Germany) embedded with a camera (QIMAGING, MicroPublisher 3.3 RTV) and connected to a computer with the software QCapturePro (Version 5.1.1.14). The magnification of the microscope was adjusted to 400x. For each replicate, 30 digital images were acquired and stored in JPEG format. The image processing was performed using the open source software ImageJ. The original images were first converted to binary (8-bit), the background substracted and particles smaller than 0.005mm tresholded (Vandamme et al., 2014). Morphological parameters were estimated through ImageJ own plugins and reported as Feret's diameter (mm) for estimation of particle size distribution (PSD). PSD of infochemicals induced flocs is reported as a histogram of the particles count against maximum Feret's diameter (mm), where each bin represents a size range used to group particles.

3.2.5 Flocculation Efficiency

Flocculation efficiency was determined by measuring the optical density (OD) of cultures before adding infochemicals and the residual OD of the supernatant after 20 h of exposure. OD readings were taken using a UV/Vis spectrophotometer (UltroSpec 3000, Pharmacia Biotech, Biochrom Ltd. Cambridge, England) at 680 nm and flocculation efficiency calculated using the following formula:

$$FE(\%) = \frac{(OD_{t0} - OD_t)}{OD_{t0}} \cdot 100$$

Eq.3-1 Flocculation Efficiency

Differences in flocculation efficiency were examined by ANOVA and posthoc Tukey test.

3.2.6 FT-IR Spectroscopic Characterization

Infrared spectroscopy (IR) measures molecular vibrations so that functional groups can be associated with characteristic infrared absorption bands, which correspond to the fundamental vibrations of the functional groups and depending on the involved types of atoms and the type/strength of chemical bonds (Berthomieu and Hienerwadel, 2009). Algal surfaces are composed of a complex, heterogeneous mixture of carboxylic, phosphoric, phosphodiester, hydroxyl and amine functional groups which all play a major role in surface binding capacity, adhesion and biofilm formation (Hadjoudja *et al.,* 2010). 11 mL aliquots were assayed from each replicate of each treatment, centrifuged and the supernatant removed. The cell pellets were air dried on the diamond of the Fourier Transform Infrared Spectrophotometer (IR Prestige-21, Shimadzu, UK). The FT-IR spectrum was read between 600 and 4000 cm^{-1} using the Happ-Genzel apodisation function over 64 scans with a resolution of 4 cm^{-1} (Mukherjee *et al.,* 2012). Microalgae cells show characteristic absorbance peaks between 970 and 1800 cm^{-1} (Dean et al., 2008), and therefore this region was used to compare cultures across different growth stage and among treatments. The software *IR solution* was used to carry out the spectral processing to remove the carbon dioxide and atmospheric water vapour and therefore reduce the noise within the spectrum. The spectra have been normalised to the intensity of a peak at 1641 cm^{-1}, corresponding to the Amide I region, with multi-point baseline correction. The data were analysed via Principal Component Analysis (PCA) to compare treatments, identify main trends and spot possible outliers. PCA is a tool which reduces the dimensionality of complex datasets while preserving their main patterns and was here used to 1)

check on the grouping of biological replicates and 2) identify the specific wavenumbers contributing to the differentiation between exposed and non-exposed algal cells to *Daphnia* infochemicals

3.3 Results

3.3.1 Composition and Growth

Upon exposure of *S. subspicatus* cells to four concentrations of *Daphnia* infochemicals (DW, DW 1:10, DW 1:100, DW 1:1000), during the early exponential phase of growth (five days of growth, ~$2 \cdot 10^6$ cells/mL), a significant increase in the mean number of colonies in the total population were observed (n=4). Treated cultures were dominated by 4- and 8-celled colonies, whereas the control was dominated by unicells (>70%) (Fig.3-2, Panel A). Surprisingly, a further analysis on independent replicates of flocs, showed that they did not consist of colonies, being instead predominantly composed of unicells (~80%) (Fig.3-2, Panel A').

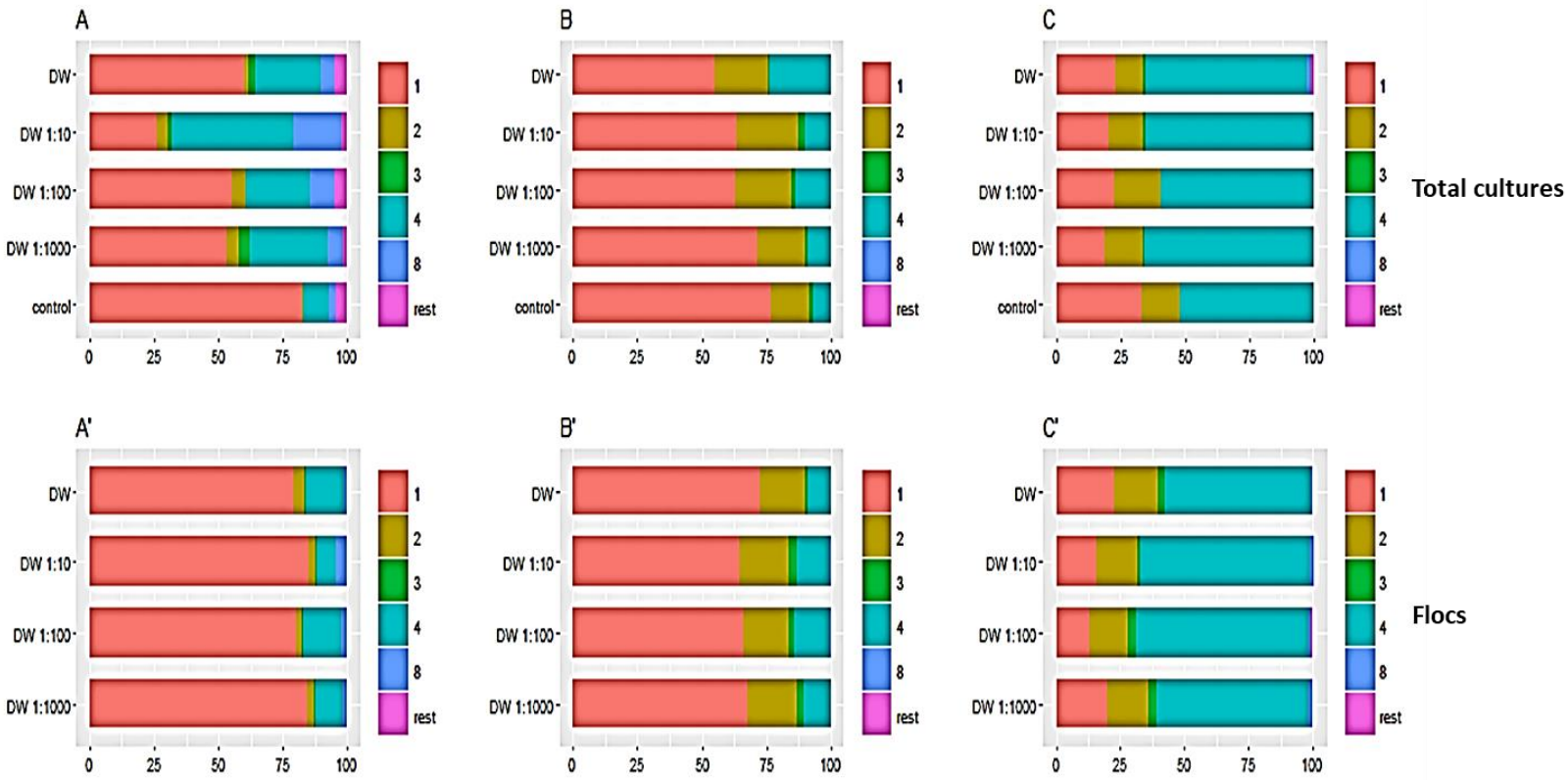


Fig.3-2 Percentages of unicells, 2-, 3-, 4- and 8-celled colonies in *S. subspicatus* populations (upper panels: A: early exponential, B: late exponential, C: stationary phase) and flocs (lower panels: A': early exponential, B': late exponential, C': stationary phase), as induced by *Daphnia* infochemicals at different concentrations. DW: undiluted Daphnia test water; DW 1:10, DW 1:100, DW 1:100: 10-fold dilutions starting from the undiluted, n=4.

When repeated during the late exponential phase (ten days of growth, ~$5 \cdot 10^6$ cells·mL) it was possible to observe an increase in the mean value of 2- and 4-celled colonies within treatments and a decrease in uni-cells, compared to control (Fig.3-2, Panel B, n=4). The analysis of flocs revealed again a predominance of unicells in all treatments (>60%) (Fig.3-2, Panel B'). During stationary phase (15 days of growth, ~$6 \cdot 10^6$ cells·mL^{-1}) no significant flocculation occurred, indicated by the formation of small particulates "debris" rather than actual flocs observed instead in the previous experiments (Fig.3-3)

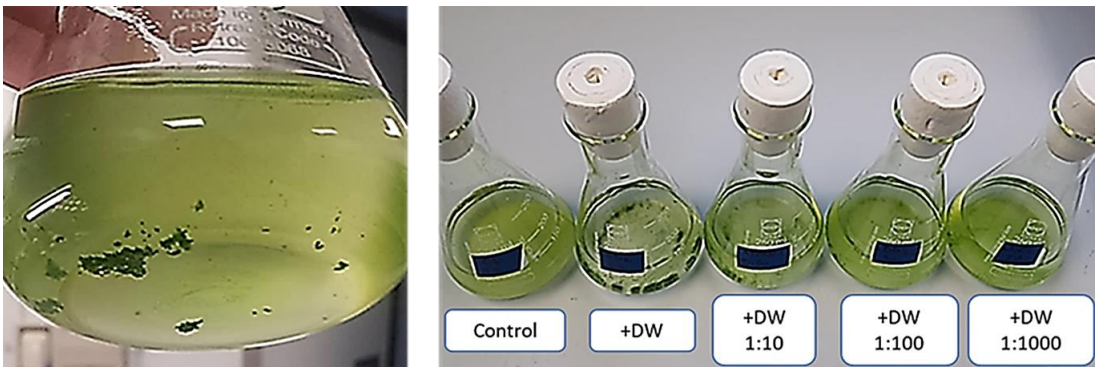


Fig.3-3 *Scenedesmus* flocs as induced by *Daphnia* infochemicals at early exponential growth stage

Within these treatments, a similar pattern of mean composition for both total cell population and debris was observed (Fig.3-2, Panel C, C', n=4). Although all treatments showed a slight decrease in the percentage of uni-cells in the total population compared to control, they were not statistically significant ($p = 0.076$). Altogether these data show infochemicals promote varying degrees of unicells and colonies distributions and flocculation efficiencies with varying dosages and algae growth stage. For each treatment and at every growth stage no significant differences among populations with and without the infochemicals test water were detected (Fig. 3-4); Vm-p = 0.534, K-p = 0.201).

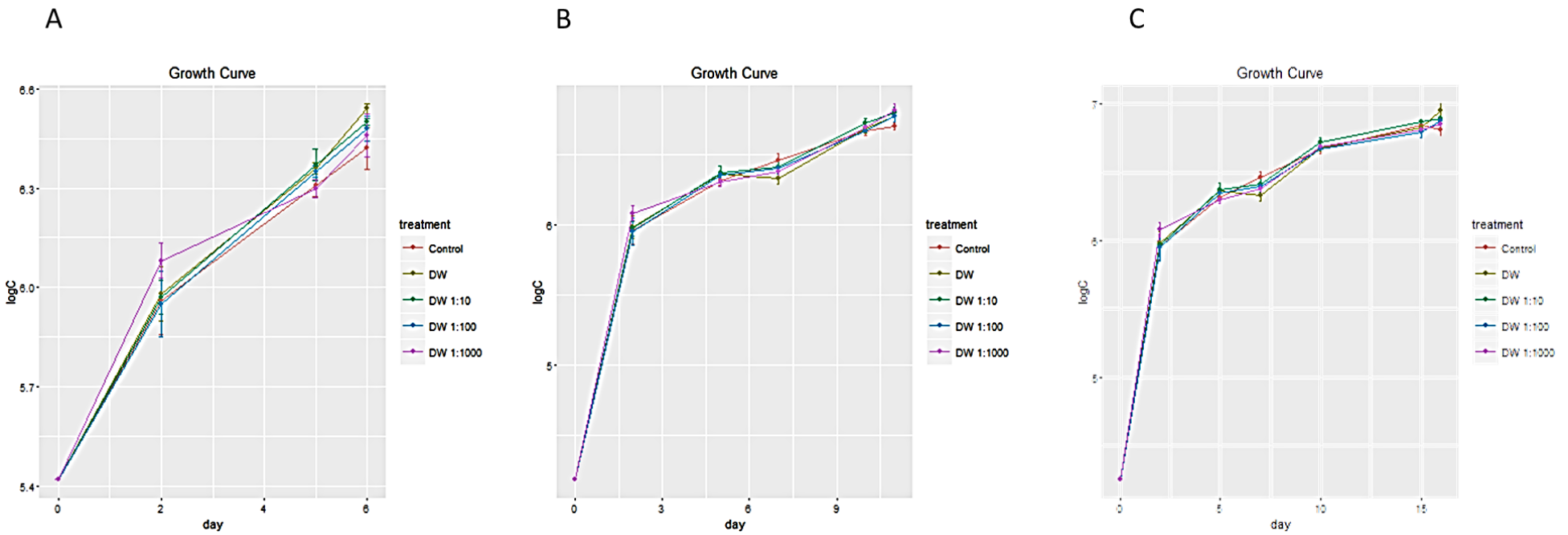


Fig.3-4 Algal growth curves after exposure to four concentrations of *Daphnia* infochemicals. Panel A: early exponential, B: late exponential, C: stationary phase

3.3.2 Morphology of Flocs

Scenedesmus flocs sampled during each harvest point and for all DW treatments were composed by assemblages of mostly unicells (Fig. 3-5), flocculating in millimetre-sized aggregates.

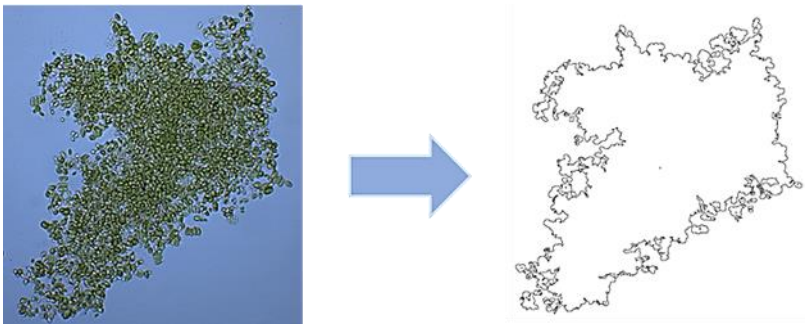


Fig.3-5 Original *Daphnia* infochemicals induced flocs (left) and binary image (right) obtained with imageJ

PSD of algae flocs changed across growth stages; aggregates were larger at early exponential (Fig. 3-6, Panel A), with a mixture of small (0-4 mm) and large flocs (4-7 mm). For all treatments, PSD shifted back towards smaller size ranges at late exponential stage (0-4 mm) (Fig.3-6, B) and comparable to control planktonic algae cells at stationary phase (0-1 mm) (Fig.3-6, C).

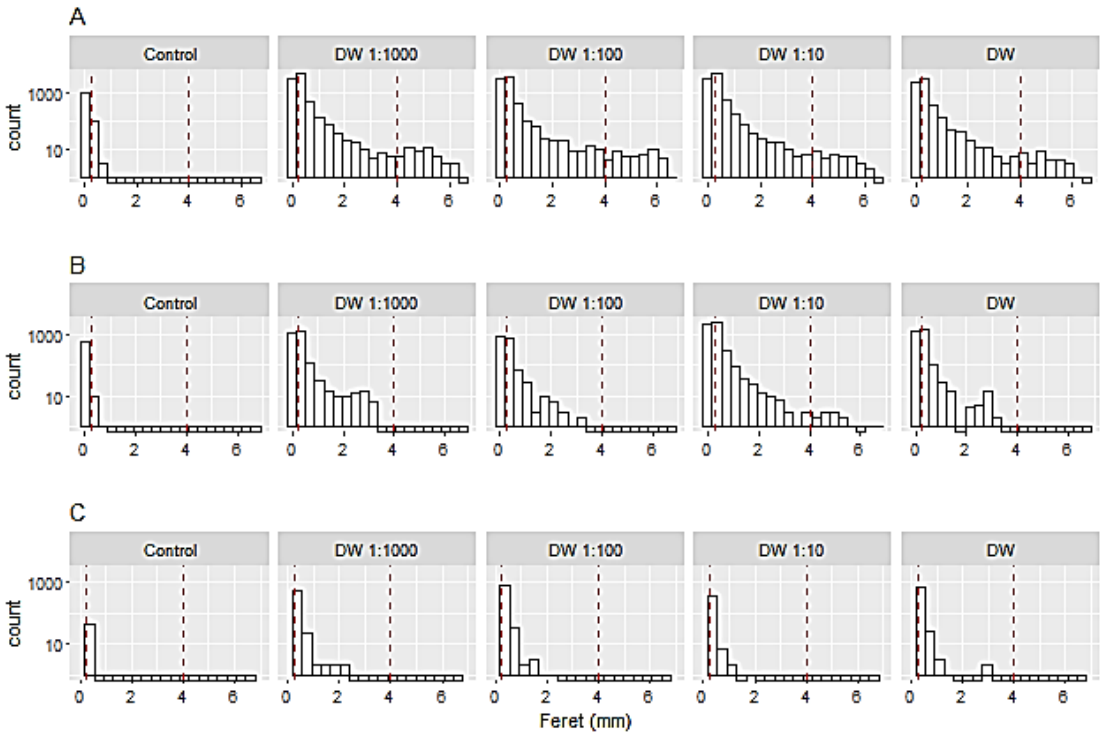


Fig.3-6 Algae floc size distributions, as induced by four concentrations of *Daphnia* infochemicals, ranging from 0 to 7 mm (Feret's diameter). Panel A: early exponential, B: late exponential, C: stationary phase

3.3.3 Flocculation Efficiency

Measured flocculation activities differed significantly between control, where no flocculation occurred, and treatments (p= 0.0016) (Fig. 3-7, Panel A), with the highest flocculation efficiency of 77.37 ± 16.93 % of algae exposed to DW. A post-hoc Tukey test revealed that only algae exposed to DW significantly differed from control (p = 0.00087). In the second experiment, algae at late exponential stage showed a lower degree of flocculation compared to early exponential and the maximum flocculation efficiency was 34.03 ± 1.32 % when algae were exposed to undiluted infochemicals (Fig.3-7, Panel B). Similarly, a post-hoc Tukey test indicated that only DW treatment significantly differed from control (p = 0.0032).

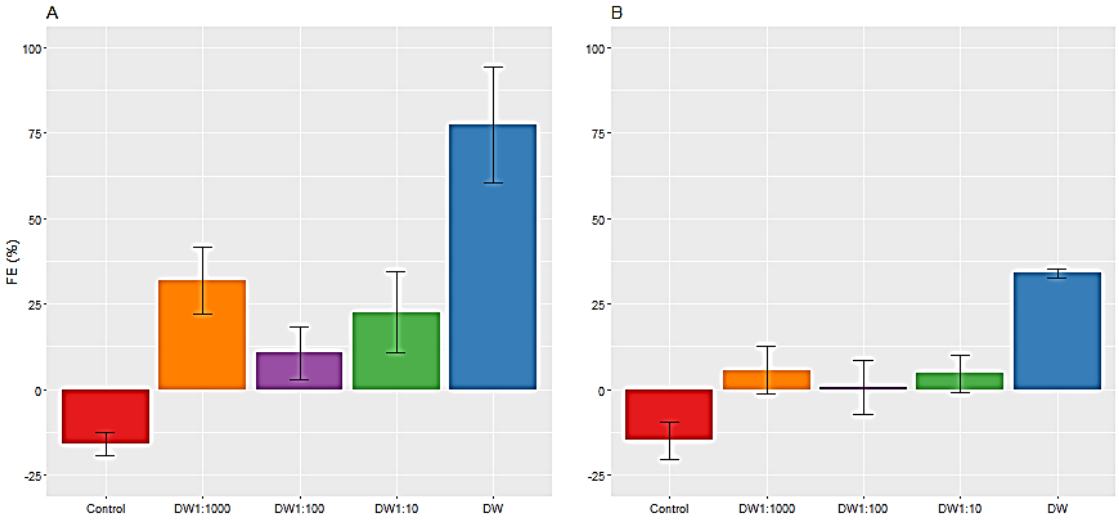


Fig.3-7 Flocculation efficiency (%) for four concentrations of Daphnia infochemicals on *S. subspicatus*. Panel A: early exponential phase, Panel B: late exponential phase

In both experiments we observed a peculiar dose-response effect. Contrarily to traditional coagulants for which an increased dosage beyond the optimum medium range value decreases the flocculation efficiency (Billuri et al., 2015, Guo et al., 2015), both early and late exponential algae cultures in our study showed an opposite trend, with the highest flocculation efficiency corresponding to the highest infochemicals concentration. In the third experiment, flocculation of algae at the stationary phase of growth was not observed for any of the treatments ($p = 0.14$).

3.3.4 FT-IR Characterization

The FT-IR spectra of S. subspicatus cells and flocs are reported in relation to growth stage (Fig. 3-8). To investigate the possible surface functional groups involved in algae-infochemicals interaction or the introduction of new peaks by the cues, the response caused in S. subspicatus by DW treatment only was analysed, as it was responsible for the highest degree of flocculation at all stages. All spectra were recorded at a pH = 7.5 ±0.5.

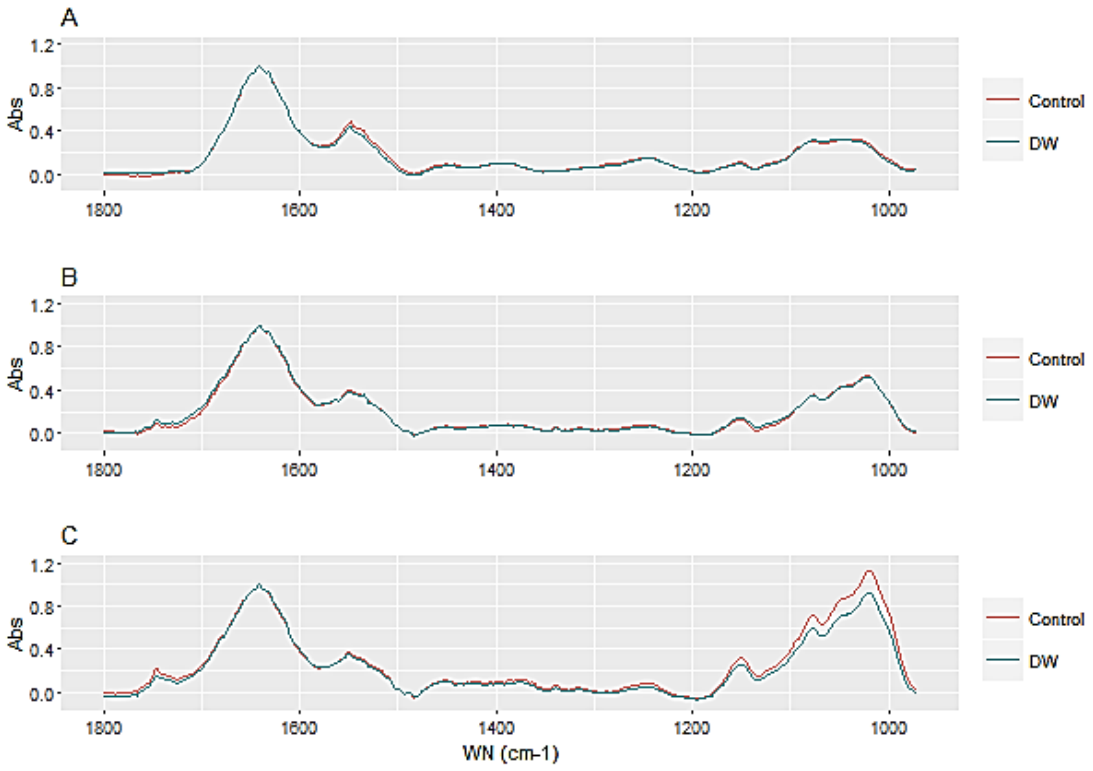


Fig.3-8 FT-IR spectra of *S. subspicatus* over the wavenumber range 970-1800 cm^{-1} sampled after 20 h of exposures of algae to *Daphnia* infochemicals. Each spectrum is a mean of three biological replicates of planktonic algae for Control, and flocculated algae for DW. Panel A: early exponential, B: late exponential, C: stationary phase

For both suspended cells and flocs at early exponential stage (Fig. 3-8, A) nine absorption peaks over the wave number range 970-1800 cm^{-1} were observed and reported in Table 3-2, according to the procedure describe by Dean et al. in 2008.

Table 3-2. *FT-IR absorption peaks and attribution of functional groups (Dean et al., 2008)*

ABSORPTION PEAK	WAVE NUMBER RANGE (cm^{-1})
Amide I ((C=O) stretching of amides from proteins)	1641
Amide II ((N-H) bending of amides from proteins)	1550
Bending of methyl from proteins (δ_{as} (CH_2) and δ_{as} (CH_3))	1400-1450 cm
Bending of methyl (δ_s (CH_2) and δ_s (CH_3)) and stretching of COO^- group (υ_s (C-O))	1380
Stretching characteristic of phosphorous molecules (υ_{as} (>P=O))	1245
Stretching of polysaccharides (υ(C-O-C))	970-1100

The same absorption bands were recorded at late exponential (Fig.3-8, B) and stationary phases (Fig.3-8, C) and an additional peak at ~1740 cm^{-1} associated with υ(C=O) stretching of ester groups from lipids and fatty acids (Dean *et al.,* 2008). Also, a distinct increase in peak intensity was recorded in the polysaccharide region (970-1100 cm^{-1}), which could be explained by the presence of glycolipids and glycoproteins on the algae cell surface. Shifts and broadening of peaks were also detected, suggesting a variation in the conformation of molecules (Wei et al., 2015). No additional peaks were detected, therefore denoting no introduction of cues into the flocs matrix, implying a possible binding or bridging mechanism for flocculation is not likely. Finally, PCA of peaks intensities showed no significant differences between control and treatments at any growth stage in the FT-IR spectra were observed (Fig. 3-9).

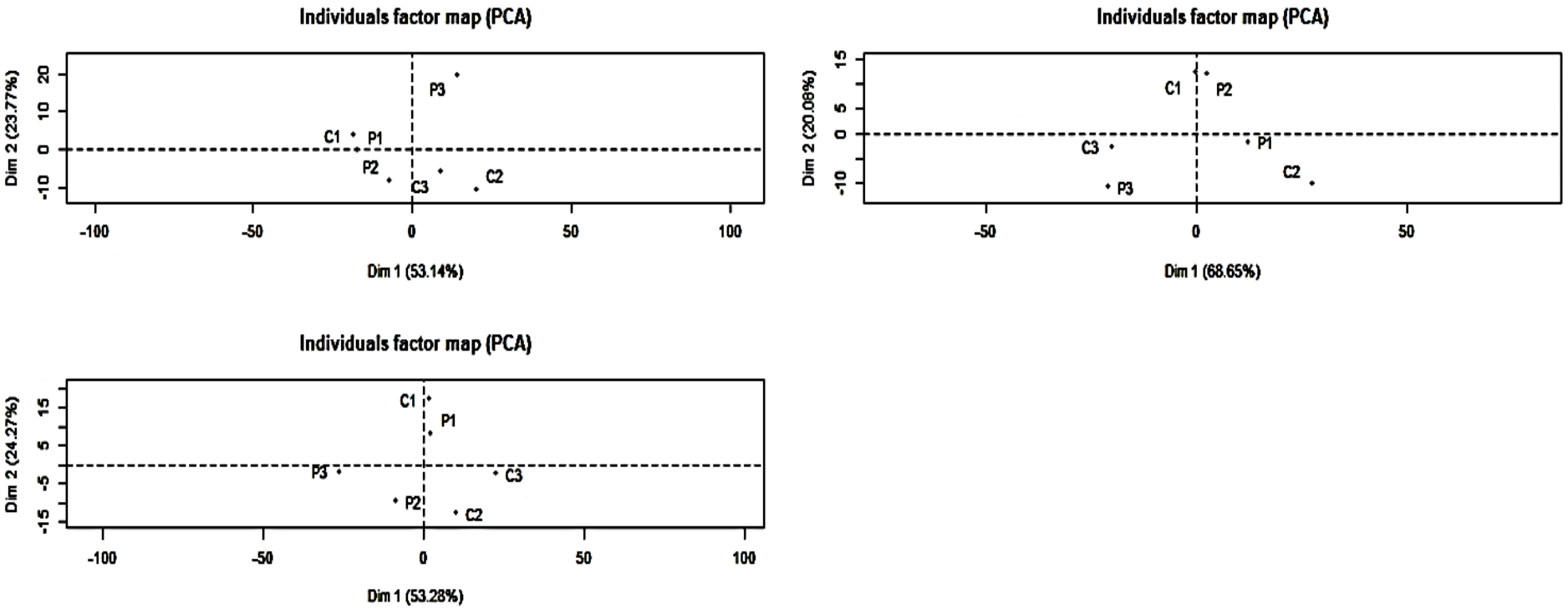


Fig. 3-9 PCA of FT-IR. Panel A: early exponential stage; Panel B: late exponential stage; Panel C: stationary stage. C: Control; P: DW, n=3.

3.4 Discussion

Daphnia infochemicals induce colony formation in *Scenedesmus* spp. as a defence against grazing. In the present study, and in accordance to literature, we found that induced defences did not affect algal growth compared to non-treated cultures (Lürling 1999, Lürling 1999a, Wu *et al.,* 2013, Zhu *et al.,* 2015). Considering the composition of cultures and flocs at all sampling stages, it could be deduced that although infochemicals induce an increase in the total amount of 2- 4- and 8-celled colonies, flocs are always principally composed by unicells, therefore suggesting a concomitant strategy of "clumping" of *S. subpspicatus*, which does not affect growth.

Also, for all non-treated cultures across the three growth stages, a decrease in the amount of unicells and an increase of colonies were observed. In the stationary phase, algal cultures were dominated by 4-celled colonies before exposure to any DW concentrations, perhaps suggesting concomitant effects causing colony formation such as nutrients deficiency (Zhu *et al.,* 2016), which occurs in batch cultures at later growth stages. This could be explained with a digestion-resistance mechanism of algal cells, according to which under nutrient limitation a shift to colonial form is due to an increase in cell volume and wall thickness to form an effective barrier against grazers digestion (van Donk and Hessen, 1993). In accordance to literature, it was observed that even at late growth stages colony formation was still stimulated by *Daphnia* infochemicals. In fact, as the generation of colonies is not a simple aggregation of cells but the result of reproduction, *Daphnia* induced colony formation occurs if cell division is not hindered (Lürling, 1999); however, results indicate that infochemicals induced flocculation is affected by several initial factors like age of the culture, initial cells concentration and initial relative distribution of unicells and colonies. The variations in

flocculation efficiency among growth stages could also be attributed to an inevitable increasing culture cell concentration across growth stages, therefore indicating that for denser cultures a higher infochemicals dosage might be required, either increasing the added amount of test water or the *Daphnia* preparation culture density. If a specific amount of infochemicals is needed per algal cell, and considering that in this study five mL of DW were shown to induce flocculation for early exponential algae at a concentration of $2 \cdot 10^6$ cells/mL, 12.5 mL would have probably been necessary for late exponential ($5 \cdot 10^6$ cells/mL) and 15 ml for stationary phase ($6 \cdot 10^6$ cells/mL) cultures. Also, algal biochemical intracellular composition varies with varying growth stages, mainly because of culture age and depletion of nutrients (Gatenby et al., 2003); cell surface characteristics too change with algal growth stage (Xia et al., 2016). These characteristics influence the efficiency of flocculation. Zhang et al., in 2012 reported in fact how the concentration of surface functional groups decreased from exponential to stationary phases; these, mostly negatively charged and dominated by carboxyl, hydroxyl and phosphoryl groups (Xia et al., 2016) are key to algal cell surface charge and suspension stability, therefore impacting algal flocculation efficiencies.

FT-IR investigation supported neither a charge neutralization – for which it would be expected that the adsorption of 'flocculants'-cues counter-ions on algal cell surface functional groups is reflected in a variations of peaks intensities, nor a bridging mechanism – which would be indicated by the presence of additional adsorption peaks coming from the flocculant structure itself (Liu et al., 2015). Therefore, alternative explanations were here investigated.

Several studies report how the production of extra polymeric substances (EPS) would affect the adhesiveness of cell surfaces, contributing to cell aggregation in some algal

species and cyanobacteria (Yang *et al.,* 2010, Harke *et al.,* 2017, Xiao and Zheng, 2016). Some authors have suggested that an increase in EPS in cells exposed to infochemicals could help explaining how *Scenedesmus* cells adhere to each other (Yang et al., 2007). In fact, EPS are heterogeneous mixture of proteins, sugars, humic substances and other important biological macromolecules which can be produced though several mechanisms, i.e. excretion, secretion, cell lysis and so on. Because of EPS high molecular weight and the presence of a variety of different functional groups, EPS can affect algal surface characteristics via electrostatic interactions and/or polymer bridging therefore greatly influencing cells aggregation (Xu et al., 2014). If infochemical induced flocculation is EPS driven, then the reason why in this study no variations were observed in any of the FT-IR spectra could perhaps be due to sample preparation used in this experimental study, as described by Karunakaran et al., 2011 and Mukherjee et al., 2012. In fact, in the case the induced EPS layer is just loosely bound to the algae cells, the centrifugation step might have caused their dispersal in the supernatant (Plude *et al.,* 1991), which was excluded from the FT-IR characterization. Therefore, in the next chapter an investigation on EPS production and characterization as induced by *Daphnia* infochemicals, and its possible role on *Scenedesmus* defence response will be presented.

3.5 Conclusions

Unravelling the mechanisms behind infochemically induced flocculation in *Scenedesmus* spp. is key to a successful application of a natural phenomenon (presence of grazers), otherwise regarded as a problem, into engineered applications like microalgal biomass harvesting. This is the first study to quantitatively assess the key parameters to consider before this approach can be applied. While growth rate

was never affected by the *Daphnia* cues, significant flocculation efficiency results were achieved for algal cultures at early exponential stage and exposed to the highest concentration of infochemicals (DW), while progressively decreasing for older cultures. This trend was also observed for PSD of flocs, with bigger flocs for algae at early exponential stage exposed to concentrated cues and smaller flocs and debris for late exponential and stationary phase cultures, respectively. Colony formation was shown to be a distinct phenomenon from flocculation, since, opposite to total cultures, flocs were predominantly composed by unicells. However, FT-IR did not show significant differences between treated and non-treated algal cultures, in terms peaks intensities and/or additional peaks. Therefore, the need to investigate the alternative hypothesis of a production of EPS responsible for aggregation of *Scenedesmus* cells.

REFERENCES – CHAPTER III

1. Alonso-Simon A, Garcia-Angulo P, Melida H, Encina A, Alvarez JM, Acebes JL. The use of FTIR spectroscopy to monitor modifications in plant cell wall architecture caused by cellulose biosynthesis inhibitors. Plant Signaling & Behavior. 2011;6(8):1104-10.

2. ASTM Materials. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Philadelphia, 1980. p. 729-80.

3. Barros AI, Goncalves AL, Simoes M, Pires JCM. Harvesting techniques applied to microalgae: A review. Renewable & Sustainable Energy Reviews. 2015; 41:1489-500.

4. Benemann JRaO, W. J. Systems and economic analysis of microalgae ponds for conversion of CO {sub 2} to biomass. Final report (No. DOE/PC/93204--T5). California Univ., Berkeley, CA (United States). Dept. of Civil Engineering; 1996.

5. Billuri M, Bonner JS, Fuller CB, Islam MS. Impact of Natural Cationic Polymers on Charge and Clarification of Microalgae Suspensions. Environmental Engineering Science. 2015;32(3):212-21.

6. Bisova K, Zachleder V. Cell-cycle regulation in green algae dividing by multiple fission. Journal of Experimental Botany. 2014;65(10):2585-602.

7. Busi S, Karuganti S, Rajkumari J, Paramanandham P, Pattnaik S. Sludge settling and algal flocculating activity of extracellular polymeric substance (EPS) derived from bacillus cereus SK. Water and Environment Journal. 2017;31(1):97-104.

8. Dean AP, Nicholson JM, Sigee DC. Impact of phosphorus quota and growth phase on carbon allocation in Chlamydomonas reinhardtii: an FTIR microspectroscopy study. European Journal of Phycology. 2008;43(4):345-54.

9. Gomaa MA, Al-Haj L, Abed RMM. Metabolic engineering of Cyanobacteria and microalgae for enhanced production of biofuels and high-value products. Journal of Applied Microbiology. 2016;121(4):919-31.

10. Guo J, Yu J, Xin X, Zou C, Cheng Q, Yang H, et al. Characterization and flocculation mechanism of a bioflocculant from hydrolyzate of rice stover. Bioresource Technology. 2015; 177:393-7.

11. Harke MJ, Jankowiak JG, Morrell BK, Gobler CJ. Transcriptomic Responses in the Bloom-Forming Cyanobacterium Microcystis Induced during Exposure to Zooplankton. Applied and Environmental Microbiology. 2017;85(4):e02832-16.

12. Hessen DO, Vandonk E. Morphological changes in Scenedesmus induced by substances released from Daphnia. Archiv Fur Hydrobiologie. 1993;127(2):129-40.

13. Jarvis P, Jefferson B, Gregory J, Parsons SA. A review of floc strength and breakage. Water Research. 2005;39(14):3121-37.

14. Karunakaran E, Biggs CA. Mechanisms of Bacillus cereus biofilm formation: an investigation of the physicochemical characteristics of cell surfaces and extracellular proteins. Applied Microbiology and Biotechnology. 2011;89(4):1161-75.

15. Lampert W, Rothhaupt KO, Vonelert E. Chemical Induction of colony formation

in a green-alga (Scenedesmus acutus) by grazers (Daphnia). Limnology and Oceanography. 1994;39(7):1543-50.

16. Lananan F, Yunos FHM, Nasir NM, Bakar NSA, Lam SS, Jusoh A. Optimization of biomass harvesting of microalgae, Chlorella sp. utilizing auto-flocculating microalgae, Ankistrodesmus sp. as bio-flocculant. International Biodeterioration & Biodegradation. 2016; 113:391-6.

17. Li M, Gao L, Lin L. Specific growth rate, colonial morphology and extracellular polysaccharides (EPS) content of Scenedesmus obliquus grown under different levels of light limitation. Annales De Limnologie-International Journal of Limnology. 2015;51(4):329-34.

18. Li T, Zhu Z, Wang D, Yao C, Tang H. Characterization of floc size, strength and structure under various coagulation mechanisms. Powder Technology. 2006;168(2):104-10.

19. Liu J, Tian Y, An X, Li G, Kang Y. Flocculation Performance and Mechanism of Removing Pectin by N-hydroxypropyl Trimethyl Ammonium Chloride Chitosan. Journal of Dispersion Science and Technology. 2015;36(11):1612-20.

20. Lurling M. The effect of substances from different zooplankton species and fish on the induction of defensive morphology in the green alga Scenedesmus obliquus. Journal of Plankton Research. 2003;25(8):979-89.

21. Lürling M. The smell of water. Grazer-induced colony formation in Scenedesmus.: Agricultural University of Wageningen; 1999.

22. Lürling M. Grazer-induced coenobial formation in clonal cultures of Scenedesmus obliquus (Chlorococcales, Chlorophyceae). Journal of Plankton Research. 1999a; 23:19-23.

23. Milledge JJ, Heaven S. A review of the harvesting of micro-algae for biofuel production. Reviews in Environmental Science and Bio-Technology. 2013;12(2):165-78.

24. Mukherjee J, Karunakaran E, Biggs CA. Using a multi-faceted approach to determine the changes in bacterial cell surface properties influenced by a biofilm lifestyle. Biofouling. 2012;28(1):1-14.

25. O'Donnell DR, Fey SB, Cottingham KL. Nutrient availability influences kairomone-induced defenses in Scenedesmus acutus (Chlorophyceae). Journal of Plankton Research. 2013;35(1):191-200.

26. Plude JL, Parker DL, Schommer OJ, Timmerman RJ, Hagstrom SA, Joers JM, et al. CHEMICAL CHARACTERIZATION OF POLYSACCHARIDE FROM THE SLIME LAYER OF THE CYANOBACTERIUM MICROCYSTIS-FLOS-AQUAE-C3-40. Applied and Environmental Microbiology. 1991;57(6):1696-700.

27. Pohnert G, Steinke M, Tollrian R. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends in Ecology & Evolution. 2007;22(4):198-204.

28. Roccuzzo S, Beckerman AP, Pandhal J. The use of natural infochemicals for sustainable and efficient harvesting of the microalgae Scenedesmus spp. for biotechnology: insights from a meta-analysis. Biotechnology Letters.

2016;38(12):1983-90.

29. Ummalyma SB, Mathew AK, Pandey A, Sukumaran RK. Harvesting of microalgal biomass: Efficient method for flocculation through pH modulation. Bioresource Technology. 2016; 213:216-21.

30. Van den Hende S, Vervaeren H, Desmet S, Boon N. Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment. New Biotechnology. 2011;29(1):23-31.

31. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

32. Vandamme D, Muylaert K, Fraeye I, Foubert I. Floc characteristics of Chlorella vulgaris: Influence of flocculation mode and presence of organic matter. Bioresource Technology. 2014; 151:383-7.

33. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology. 2013;31(4):233-9.

34. Wei Z, Jiao D, Xu J. Using Fourier Transform Infrared Spectroscopy to Study Effects of Magnetic Field Treatment on Wheat (Triticum aestivum L.) Seedlings. Journal of Spectroscopy. 2015.

35. Wijffels RH, Kruse O, Hellingwerf KJ. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Current Opinion in Biotechnology.

2013;24(3):405-13.

36. Wu X, Zhang J, Qin B, Cui G, Yang Z. Grazer density-dependent response of induced colony formation of Scenedesmus obliquus to grazing-associated infochemicals. Biochemical Systematics and Ecology. 2013; 50:286-92.

37. Xiao R, Zheng Y. Overview of microalgal extracellular polymeric substances (EPS) and their applications. Biotechnology Advances. 2016;34(7):1225-44.

38. Xu H, Jiang H, Yu G, Yang L. Towards understanding the role of extracellular polymeric substances in cyanobacterial Microcystis aggregation and mucilaginous bloom formation. Chemosphere. 2014; 117:815-22.

39. Yang Z, Kong F, Shi X, Xing P, Zhang M. Effects of Daphnia-associated infochemicals on the morphology, polysaccharides content and PSII-Efficiency in Scenedesmus obliquus. International Review of Hydrobiology. 2007;92(6):618-+.

40. Yang Z, Liu Y, Ge J, Wang W, Chen Y, Montagnes D. Aggregate formation and polysaccharide content of Chlorella pyrenoidosa Chick (Chlorophyta) in response to simulated nutrient stress. Bioresource Technology. 2010;101(21):8336-41.

41. Zachleder V, Bisova K, Vitova M, Kubin S, Hendrychova J. Variety of cell cycle patterns in the alga Scenedesmus quadricauda (Chlorophyta) as revealed by application of illumination regimes and inhibitors. European Journal of Phycology. 2002;37(3):361-71.

42. Zhu X, Nan H, Chen Q, Wu Z, Wu X, Huang Y, et al. Potential grazing intensity

directly determines the extent of grazer-induced colony formation in Scenedesmus obliquus. Biochemical Systematics and Ecology. 2015; 61:271-7.

43. Zhu X, Wang J, Chen Q, Chen G, Huang Y, Yang Z. Costs and trade-offs of grazer-induced defenses in Scenedesmus under deficient resource. Scientific Reports. 2016;6.

CHAPTER IV

Effects of Daphnia Infochemicals on Production and Distribution of EPS in Scenedesmus subspicatus

4.1 Introduction

EPS are macromolecular compounds secreted by microbial cells (algae, bacteria, cyanobacteria) during growth and are composed of a complex high-molecular-weight mixture of biopolymers with various structure and diverse composition (Xu et al., 2014). Primary components include carbohydrates, proteins, uronic acids and lipids but nucleic acids and inorganic compounds can also be found. The range of these molecules and their relative abundance can vary under the influence of several factors including algal species and strain, age of the culture or environmental conditions including nutrient status, temperature, pH, salinity (Xiao and Zheng, 2016). Many functions have been attributed to EPS, including adhesion of cells, protection against grazers or toxic substances and binding to metals (Whitton and Potts, 2007), which result in the formation of algal aggregates, a process known as bio-flocculation. Many investigations, both in the field of wastewater treatment and algal research, have been performed to investigate the role of EPS in bio-flocculation. The aggregrates or flocs possess different physicochemical properties like structure, viscosity, surface charge, flocculation and settling, then freely suspended cells (Xiao and Zheng, 2016). However, research evaluating the role of EPS in bio-flocculation are often contrasting, showing either a positive correlation between EPS content and bio-flocculation or negative or no correlation at all (Li and Young, 2007, Mannheim and Nelson, 2013, Shen et al., 2014, Jakob et al., 2016). The composition of EPS, as well as the relative proportion of EPS components, have been indicated to be more important than quantity when inducing flocculation (Wilen et al., 2003, Li and Young, 2007), as in some cases an increased production of EPS is not linked to higher flocculation efficiencies, while a higher abundance of hydrophobic groups from proteins, humic substances or uronic acids has been shown to contribute to aggregate stability and

enhancement of flocculation (Wilen et al., 2008, Guo et al., 2016). In fact, as EPS 'glues' cells together by either electrostatic forces, bridging by cations, entanglement of EPS molecules or hydrophobic interactions, and the stability of these aggregates complies with the general rules for colloidal chemistry (see Chapter I), any variation in physico-chemical properties can influence the inter particle forces between the floc constituents (Wilen et al., 2008).

Induced flocculation is an established technology for algal biomass harvesting. It can be achieved using several methods, including charge neutralization by metal salts, one of the most commonly employed methods (Alam et al., 2016). The main drawback to this approach is the build-up of metals in the system which cause contamination of both biomass and growth medium therefore requiring post-processing of the biomass (i.e. feed) and constraints on recycling of the medium in the system (Vandamme *et al.,* 2013). Other methodologies which do not contaminate the biomass depend on the use of polymers like chitosan or physical methods, i.e. centrifugation or electromagnetic pulses. These however are more expensive and difficult to be applied on a large scale (Vandamme *et al.,* 2013). Variations in culture temperature or pH can also induce algal flocculation, but these processes can lead to undesirable changes in cell composition (Benemann & Oswald, 1996), such as alterations in the saturation degree of fatty acids in the cell membranes or the starch content (Juneja et al., 2013).
A growing interest is being shown in bio-flocculation methods, including the microbial production of EPS as flocculants (Wang et al., 2018). However, bio-flocculation has often been considered too species-specific, slow or unreliable (Milledge and Heaven, 2013).

In this study, the focus is on EPS production induced by infochemicals released by the grazers *Daphnia* spp generating colony formation and flocculation (see Chapter III) in *Scenedesmus* spp. While colony formation can be defined as a cell division process producing binary multiples of cells joined by a common cell wall, flocculation is tied more closely to aggregation, where charge changes and/or EPS act as a glue to bind uni-cells (see Chapter III). If flocculation is driven by EPS production, then the amount of protein, carbohydrate or uronic acids groups in the total culture should vary (Yang et al., 2007) or the individual chemical composition and structure change to reduce repulsion among cells. In fact, specific EPS constituents can play a determinant role in cell aggregation, either promoting or hampering flocculation (Badireddy et al., 2010). This is because several intermolecular interactions and their net balance can contribute to aggregation of cells. Generally, these are the DLVO-type interactions (see Chapter I) but also bridging of EPS via positively charged ions, hydrophobic and steric interactions between long-chain EPS molecules (Wilen et al., 2003).

In the present work, the induced EPS production in *S. subspicatus* cultures at early exponential stage after exposure to *Daphnia* infochemicals was experimentally explored. The focus is on the assessment of soluble EPS (sEPS) of *Scenedesmus* cells and flocs, and relative abundance of sugars, proteins and uronic acids, employing several methods. Firstly, negative staining was used to visualise and compare planktonic cells versus cells in flocs. Secondly, EPS were extracted using established protocols and the material subjected to standard assays for protein (Lowry), carbohydrate (phenol-sulphuric acid) and uronic acids (modified carbazole method). Detailed descriptions of these methods are provided in Appendixes I-IV, along with comparisons of methods and motivations behind their selection. The experimental

design was slightly modified from the previous experiments reported in this thesis as it was important to distinguish between the effects induced by infochemicals from those of its carrier into the algal culture, i.e. ASTM water which is produced by combining distilled water with four salts ($MgSO_4 \cdot 7\ H_2O$, $NaHCO_3$, KCl, $CaSO_4 \cdot 2\ H_2O$). In fact, salinity in a wide range of concentrations is one of the factors reported in literature to contribute to EPS production in microalgae as cellular protection mechanism (Mishra and Jha, 2009).

4.2 Experimental Design

Five mL of exponentially growing *S. subspicatus* (~$2 \cdot 10^6$ cells/mL) were transferred to 250 mL Erlenmeyer flasks containing 150 mL of autoclaved Ebert's medium and incubated for five days (early exponential stage) at 20 ±1 °C on a shaking table at 120 rpm, continuously illuminated from above by light tubes at 259 µmol/ m^2s and randomly rearranged every day. On day five, algae were treated with either five mL of additional Ebert's medium (control), DW or ASTM water, to investigate the possible side effects of the presence of ASTM salts on EPS production in *S. subspicatus*. Sampling was performed after 2h and 20h of exposure to observe variations early enough under infochemicals effects and at a time point after which no further flocculation is observed (as noted in previous experiments). Cultures were centrifuged at 4000g for 15min at 4°C to extract sEPS. The supernatant fraction was filtered through a 0.22 µm GV, PVDF membrane, dialysed against ten litres of distilled water per cycle (for a total of six water changes) at 6°C using a membrane (SnakeSkin Dialysis Tubing, 3.5k MWCO, Thermo Scientific), freeze dried and re-suspended in 500 µl HPLC grade water for further quantification assays. The experimental workflow is reported in Fig. 4-1.

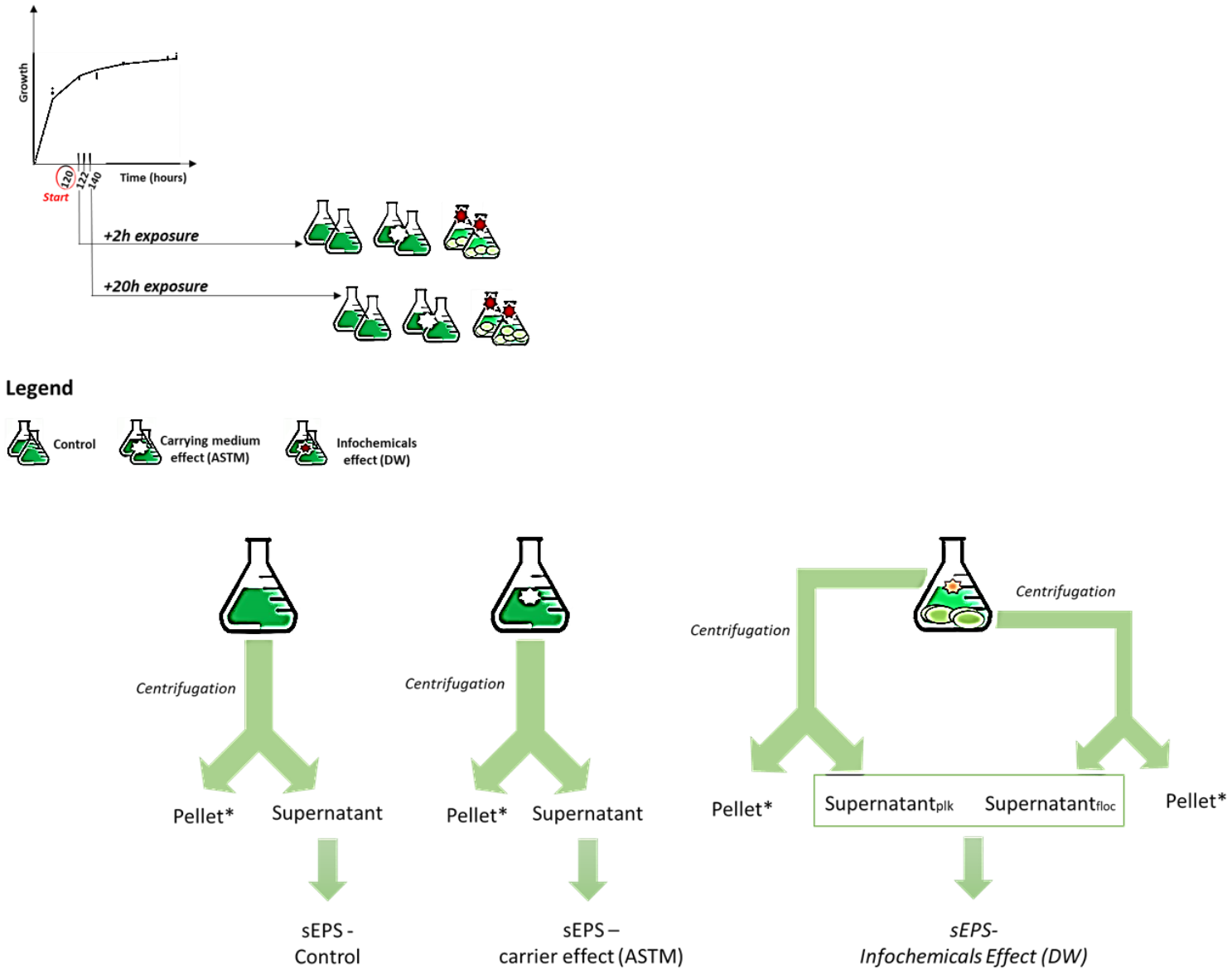


Figure 4.1 Experimental workflow for the analysis of sEPS

4.3 Methods

4.3.1 Negative Staining

A small drop of a 2% solution of Nigrosin (Sigma-Aldrich) was placed on the edge of a clean microscope slide and mixed with the same volume of algal flocs. Another slide was then placed against the suspension at a 45° angle to spread the drop along its edge. The formed smear was air dried and examined under a microscope (Leitz Wezler, Germany) at 400x magnification.

4.3.2 Carbohydrates

sEPS-carbohydrates were analysed using the phenol-sulphuric acid assay. 100µL of sample/ glucose standards were mixed with 100µL of freshly prepared 5% (v/v) phenol in water, followed by addition of one mL of sulphuric acid. Samples/glucose standards were incubated for five minutes at 90°C and then cooled down to room temperature. Absorbance readings were taken at a wavelength of 495 nm using the 0 µg/ml standard as blank. Two biological replicates, with three technical replicates each, were used and results normalized by sEPS dry weight.

4.3.3 Proteins

sEPS- proteins were quantified using the Lowry assay. 300µl of samples/standards were mixed with 700µl of water. To each one ml sample/standard 100 µl of 0.15% (w/v) sodium deoxycholate was added and incubated for ten minutes at room temperature. 100µl of 72% TCA were added, followed by centrifugation at 3000 g for 15 minutes at room temperature. Supernatants were discarded and pellets air dried for 30 minutes. Pellets were re-suspended in 500 µl of milliQ water, then added with a freshly prepared solution of 0.8M NaOH, 10% SDS, Copper tartrate carbonate solution (CTC) (0.2% Potassium sodium tartrate tetrahydrate, 0.1% Copper Sulphate, 10%

Sodium Carbonate) and water in a 1:1:1:1 ratio and finally incubated at room temperature for ten minutes. 250µl of Folin's reagent (1:6 diluted) were added and incubated for 30 minutes for colour to develop. Absorbance readings were taken at 750nm with a Jenway 7315 spectrophotometer, using the 0 µg/ml standard as blank. Two biological replicates, with three technical replicates each, were used and results were normalized by sEPS dry weight

4.3.4 Uronic Acids

Uronic acids in sEPS were analysed using a modified carbazole. All chemicals were purchased from Sigma-Aldrich and solutions prepared in HPLC grade water. One mg/mL stock solution of D-Glucuronic Acid was freshly prepared and used to build calibration curves over the range 0-20 µg/mL. 20 µl of 4 M potassium sulfamate were added to 200 µl of samples/standards, followed by the addition of 1.2 ml of concentrated sulphuric acid. To each tube, 40 µl of solution composed by 0.15% m-hydroxy-diphenyl in 0.5% sodium hydroxide were added and incubated for 15 minutes at room temperature to allow colour development (pink). Absorbance readings were taken at 525 nm using a Jenway 7315 spectrophotometer, with the 0 µg/ml as blank. Analysis relied on the use of two biological replicates, with three technical replicates each and then normalized by sEPS dry weight.

4.4 Results

4.4.1 Negative Staining

Microscopic examination of negatively stained *S. subspicatus* flocs revealed a non-uniform distribution of an alleged EPS layer surrounding the cells and accumulation in the inner parts of the flocs (Fig. 4-2)

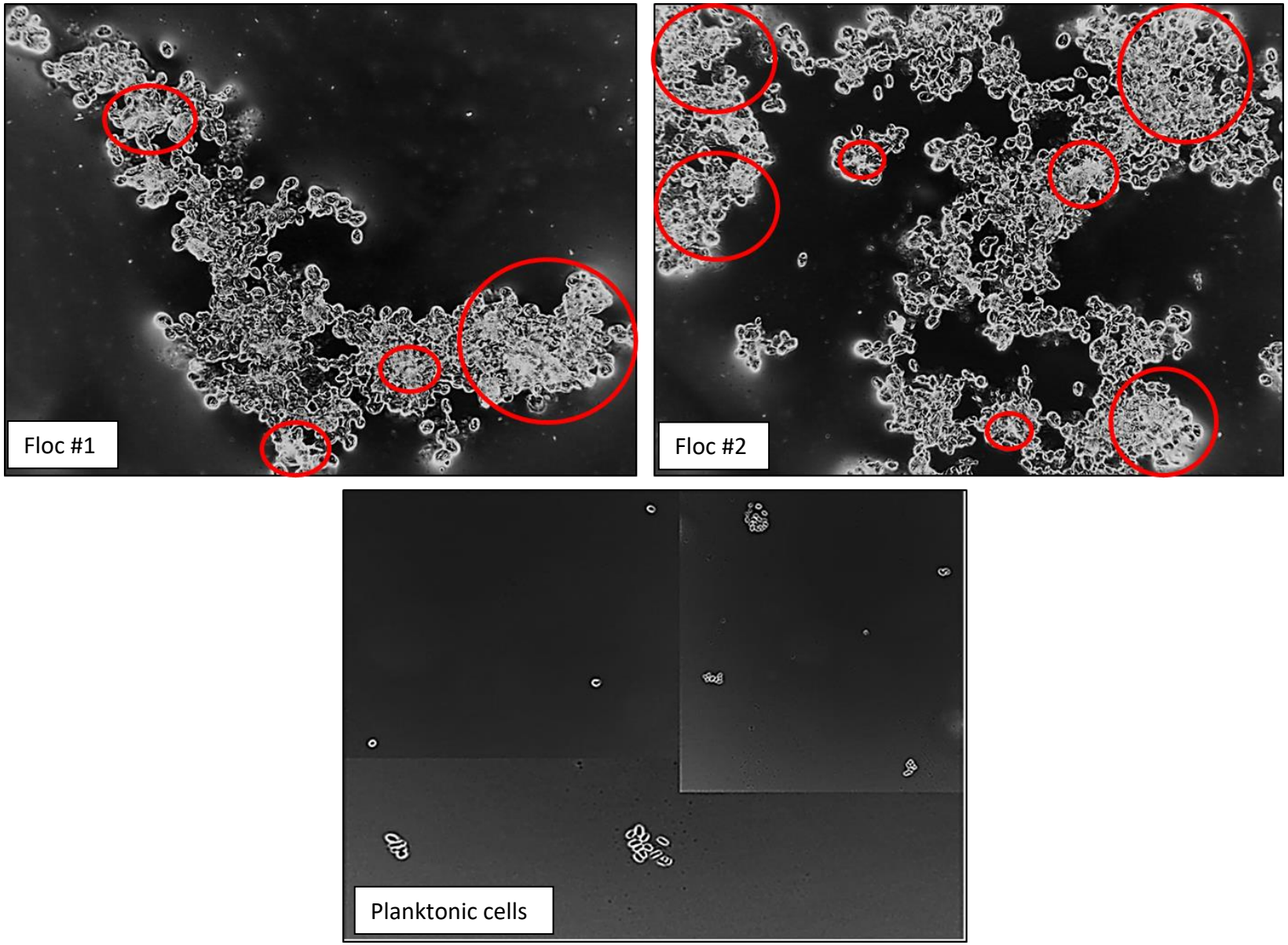


Fig.4-2 Negative stained planktonic *Scenedesmus* cells- Control (lower panel) and flocs (upper panel).

Although visual inspection seemed to indicate the presence of EPS surrounding cells and accumulating in the inner part of *S. subspicatus* flocs, further and more in-depth investigations were required to confirm the hypothesis of an induced production of sEPS as responsible for aggregation of microalgal cells in response to predation cues.

4.4.2 sEPS

Figure 4-3 shows variation in carbohydrates, proteins, uronic acids content in sEPS relating to specific time of exposure of *S. subspicatus* to info-chemicals: early (2h) and late (20h).

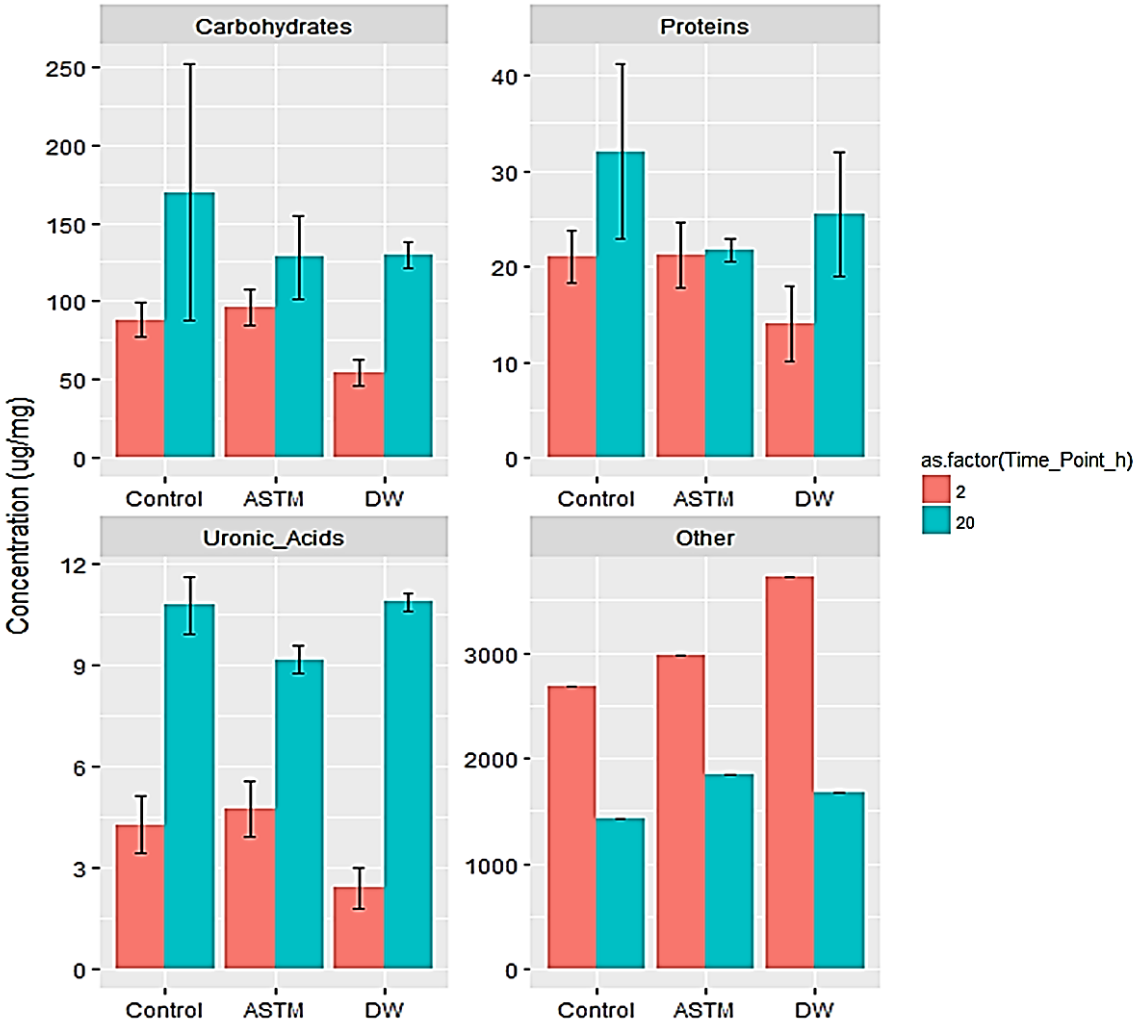


Fig. 4-3 sEPS in *S. subspicatus* after exposure to *Daphnia* infochemicals

No significant difference in any of the sEPS components under study was found between controls and treatment and at both time points of interest. Only exception was represented by the "other" fraction, calculated as the difference between the total EPS dry weight and the sum of the sugars/proteins/uronic acids amounts. In fact, the other fraction was the the dominant part of sEPS, with higher concentrations for DW treatment, i.e. algae exposed to infochemicals (range 2.800-3.500 μg/mg of EPS dry weight).

Subsamples were also sent to the University of Huddersfield, analysed by NMR spectroscopy and hypothesised as small molecules, remnants of lipid based materials (courtesy of Professor Andrew Laws).

4.5 Discussion

Previous studies have suggested that flocculation of algae and cyanobacteria was related to the production of EPS. For example, Yang et al. in 2007 investigated the effects of infochemicals from the grazer *D. carinata* on colony formation and polysaccharides content in the microalga *Scenedesmus obliquus*. Authors reported a simultaneous increase in the number of colonies and the total polysaccharides content in *S. obliquus* cultures exposed to infochemicals in comparison to non-exposed cultures, indicating that *Daphnia*-associated infochemicals boost the synthesis of extracellular polysaccharides in *S. obliquus* and play an important function in cells adhesion. Later in 2010, Yang et al. studied the role of nutrients stress on the stimulation of extra-polysaccharides production and its relation to *Chlorella pyrenoidosa* aggregates size, revealing that the generation of aggregates and increase in polysaccharides concentrations occurred at the same time therefore suggesting their role in joining cells together. Authors observed no impact on microalgal growth rate; also, the aggregates were formed by a random distribution of cells, hence reporting the phenomenon

as due to adhesion of already existing free cells with polysaccharides working as a sticky matrix. Li et al., in 2013 investigated the effects of linear alkylbenzene sulfonate (LAS), an anionic surfactant with a chemical structure similar to that reported for *Daphnia* infochemicals (Yasumoto et al., 2008) on extracellular polysaccharide content and cells per particle of *Microcystis aeruginosa* and *S. obliquus*, to find a positive relation between EPS-sugars content and cells per colony in both species, not clearly distinguishing however between colonies (coenobia) or aggregates. In 2017, Harke et al. investigated the transcriptomic responses of *M. aeruginosa* upon exposure to infochemicals from two *Daphnia* species, i.e. *D. magna* and *D. pulex*, to find an increased transcript abundance of genes regulating EPS-sugars production and export (glycosyl transferases, sugar modification enzymes, outer membrane porins and polysaccharide export protein) and which were associated with colony formation as a deterrent mechanism against predation.

Despite grazer induced colony formation in *Scenedesmus* spp being widely documented (Hessen and van Donk 1993, Lampert *et al.,* 1994, Lürling 1999, Lürling 1999a, Lürling 2003, van Holthoon *et al.,* 2003, Pohnert *et al.,* 2007, O'Donnell *et al.,* 2013, Wu *et al.,* 2013, Zhu *et al.,* 2015), the actual molecular mechanism is still largely unexplained. Based on previous research on both microalgae and cyanobacteria (Yang *et al.,* 2007, Yang et al., 2010, Li et al., 2013, Harke et al., 2017), it was here hypothesised that an increase in either total sEPS or variations in the relative distribution of its components after exposure to *Daphnia* infochemicals could be a trigger for flocculation and/or colony formation in *S. subspicatus*. In fact, the relative proteins/carbohydrates ratios can affect the hydrophobic character of EPS and therefore cells aggregation and flocculation (Quigg et al., 2016). Also, the presence in the EPS of acidic polysaccharides like uronic acids can facilitate flocculation, as their

carboxyl groups provide effective sites for the attachment of cells (More et al., 2012, Zhong et al., 2014). Although similar extraction methods (centrifugation followed by dialysis) with slightly different operating parameters such as centrifugation speed, as well as quantification assays to the above-mentioned studies were here employed, the present findings did not confirm the initial assumption of an increased EPS production or of a redistribution of those individual primary components, i.e. polysaccharides, proteins and uronic acids. However, in 2016 Li and Gao also reported how stress induced colony formation in *S. obliquus* was not linked to an increase in EPS algal content. Authors pointed instead on the so-called ultrastructure of *Scenedesmus* (Pickett-Heaps and Staehelin, 1975), with cells connected by a layer of material in the gap between the continuous trilaminar sheath and the ornamented layer of this microalga, and whose composition is "pectic", i.e. polysaccharides (D'Hondt et al., 2018) but could not be regarded as *conventional* EPS, thus suggesting to rather investigate on the regulation of gene expression for the layer of connected cells. The role of EPS components on algal flocculation other than the most commonly studied proteins and polysaccharides is not well established yet, although their hydrophobic and/or hydrophylic features can considerably affect the process. The presence of the significant portion of the other fraction in the sEPS suggests further investigations would be needed. Although not quantified in this thesis work, there are indications that the other EPS fraction could consist of lipids-based components. Other studies have also reported the presence of other fractions in the EPS complex in activated sludge and which were hypothesised as lipids (Liu and Fang, 2002). Future research should focus on the analysis of lipids components in the EPS, i.e. fatty acids and lipo-polysaccharides, which affect hydrophobic/hydrophilic properties of cell surface and consequently impacting aggregation mechanisms (Al-Halbouni et al.,2008, Flemming and Wingender, 2010). Finally,

the extraction method used might not have efficiently extracted all the EPS fractions from the algal culture and further investigations should also evaluate alternative methods to fully characterize the *Daphnia* induced flocculation in *S. subspicatus*.

4.5.1 Extraction Methods and Bound EPS

Extraction methods greatly influence EPS quantification (Liu and Fang, 2002, Hong et al., 2017) and to date there is no standard established procedure. Also, during extraction the disruption of macromolecules as well as the lysis of cells can occur, although its extent is difficult to evaluate (Sheng et al., 2010). EPS are usually divided into two classes: 1) soluble EPS (sEPS), the focus of this investigation, which remain in the supernatant after centrifugation and 2) bound EPS (bEPS), which instead compose the pellet after the centrifugation step (Liang et al., 2010, Maqbool et al., 2015, Zhang et al., 2016). bEPS are further subdivided into 1) tightly bound (TB-EPS), which are bound to the cell surface in a tight and stable way and 2) loosely bound (SB-EPS), which are loose and dispersible (Guo et al., 2016). Guo et al., 2016 reported that TB-EPS are independent of the formation of flocs, and Cai and co-workers in 2016 also reported that LB-EPS negatively influence bio-flocculation. Moreover, from FT-IR characterization of algal cells exposed to infochemicals (see Chapter III) there was no indication of variations in the cell surface functional groups which might have suggested changes in terms of bEPS. Also, to achieve an accurate description of each fraction, hence elucidating their role in microalgal bio-flocculation, there is a need to improve bEPS extraction method and without contamination due to internal components (Takahashi et al., 2009). However, there is not an easy way to extract all EPS and the chosen technique must be selected and fine-tuned for each case under study, considering it might be necessary to combine and repeat extraction steps for the full recovery of the various EPS fractions (Sheng et al., 2010).

4.6 Conclusions & Future Directions

Based on results reported, it could be concluded that sEPS production might be responsible for the infochemicals induced colony formation and flocculation in *Scenedesmus subspicatus*. However, further investigations are needed to look into the composition and relative distribution of the other fraction to unravel the presence of lipids and a possible re-distribution of secreted substances responsible for colony formation and aggregation.

In any case, production of EPS requires a supply of precursors, which should be reflected in variations in cellular metabolism. On this basis, the next chapter will describe a proteomic analysis of *S. subspicatus* cells, where changes in protein abundances can provide insight into metabolic changes that occur in response to infochemicals exposure.

APPENDIX I: NEGATIVE STAINING

Negative staining is an easy and inexpensive technique that involves the use of an acidic stain such us Nigrosin or India ink. Being characterised by negatively charged chromogen, it does not penetrate the algal cells because of the negative charge on their surface therefore facilitating visualization of unstained layers/structures against a coloured background. However, the appropriate stain concentration is to be determined via a trial and error procedure (Cullimore, 2008). Algal EPS can be visualized with light microscopy after negative staining in the form of a white layer surrounding the cell. (Schmid *et al.,* 2016).

APPENDIX II: POLYSACCHARIDES

Despite the significant development of several analytical techniques, colorimetric methods are still the most simple and cheap procedures for quantitative determination of total carbohydrates and are commonly used and universally accepted (Le and Stuckey 2016). Most involve the use of sulphuric acid and a reagent to develop colour such as anthrone (Dreywood, 1946) or phenol (Dubois 1956). However, they are time consuming and not specific and the results are reported in terms of a standard-equivalent concentration, usually glucose. This might result in under or over estimations in cases where the carbohydrates composition in the sample is not well known and variable responses to other than glucose saccharides are observed (Le and Stuckey 2016). Previous studies have reported that *Scenedesmus* species EPS sugars fraction consists of hexoses and pentoses (Guo *et al.,* 2013) therefore screen assays feasibility and performance were screened towards glucose, mannose (C6) and xylose (C5) as standards.

II-I ANTHRONE ASSAY

Anthrone is a tricyclic aromatic ketone ($C_{14}H_{10}O$) which reacts with saccharides to form a blue-green complex (Dreywood, 1946). Sulphuric acid and heat cause the hydrolysis of glycoside bonds of polysaccharides and dehydration of monosaccharides to produce furfural compounds which then react with anthrone to produce a coloured product whose absorbance can be measured using a spectrophotometer. The anthrone -sulphuric acid solution should be prepared freshly because it is light sensitive and its absorption decreases over time (Le and Stuckey 2016).

Here, all chemicals were purchased from Sigma-Aldrich and solutions prepared in HPLC grade water. One mg/mL stock solutions of glucose/xylose/mannose were freshly prepared and used to build calibration curves over the range 0-200 µg/mL. 400µL of sample/standards were mixed with 800µL of anthrone - sulphuric acid solution and incubated in the dark at 80°C for 30 minutes. Once sample/standards were cooled to room temperature, absorbance measure are taken at a wavelength of 625 nm with a Jenway 7315 spectrophotometer, using the 0 µg/ml standard as blank.

II-II Phenol-Sulphuric Acid Assay

Phenol in the presence of sulfuric acid can be used for the quantitative colorimetric determination of polysaccharides. The assay is simple, rapid and sensitive and gives reproducible results using a cheap and stable reagent (Dubois *et al.,* 1956). Full description of the method is given in Paragraph 4.3.2. As in the anthrone assay, heat and acidic environment induce hydrolysis of polysaccharides followed by dehydration of monosaccharides and production of furfural derivatives which react with phenol to form complexes with a characteristic orange-yellow colour. While hexoses produce hydroxy-methyl-furfuraldehyde and methyl-furfuraldehyde, pentoses react to form furfuraldehyde (Bailey, 1957). It was noticed that the response of xylose to anthrone was less colourful and instable across reagent concentrations, if compared to hexoses. (Fig. II-1) This could be explained by a reaction between the furfuraldehyde-anthrone complex which is formed and excess of anthrone, while the methyl group present in the furfuraldehyde derivatives may prevent or considerably slow down such a reaction (Bailey, 1957). Also, at both anthrone concentrations under study (0.1% and 0.2% w/v), glucose displayed signal saturation at

concentrations greater than 100 µg/ml and mannose gave a lighter yet linear response over the concentration range screened.

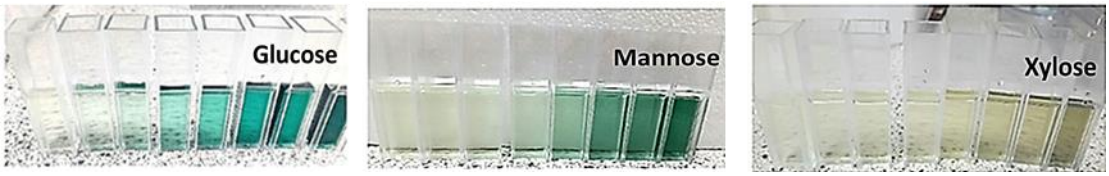


Fig.II-1 Responses of Glucose, Mannose and Xylose with Anthrone 0.1% in sulfuric acid (w/v)

The reason why sugars with a similar chemical structure gave a different response could be explained by slight differences in the wavelength value for maximum absorbance and/or inconsistencies in the colorations of the furfural derivatives. Phenol-sulphuric acid assay proved to be more reliable, with good linearity observed for all the sugars under study (Fig II-2) and therefore selected for further analyses with glucose as standard.

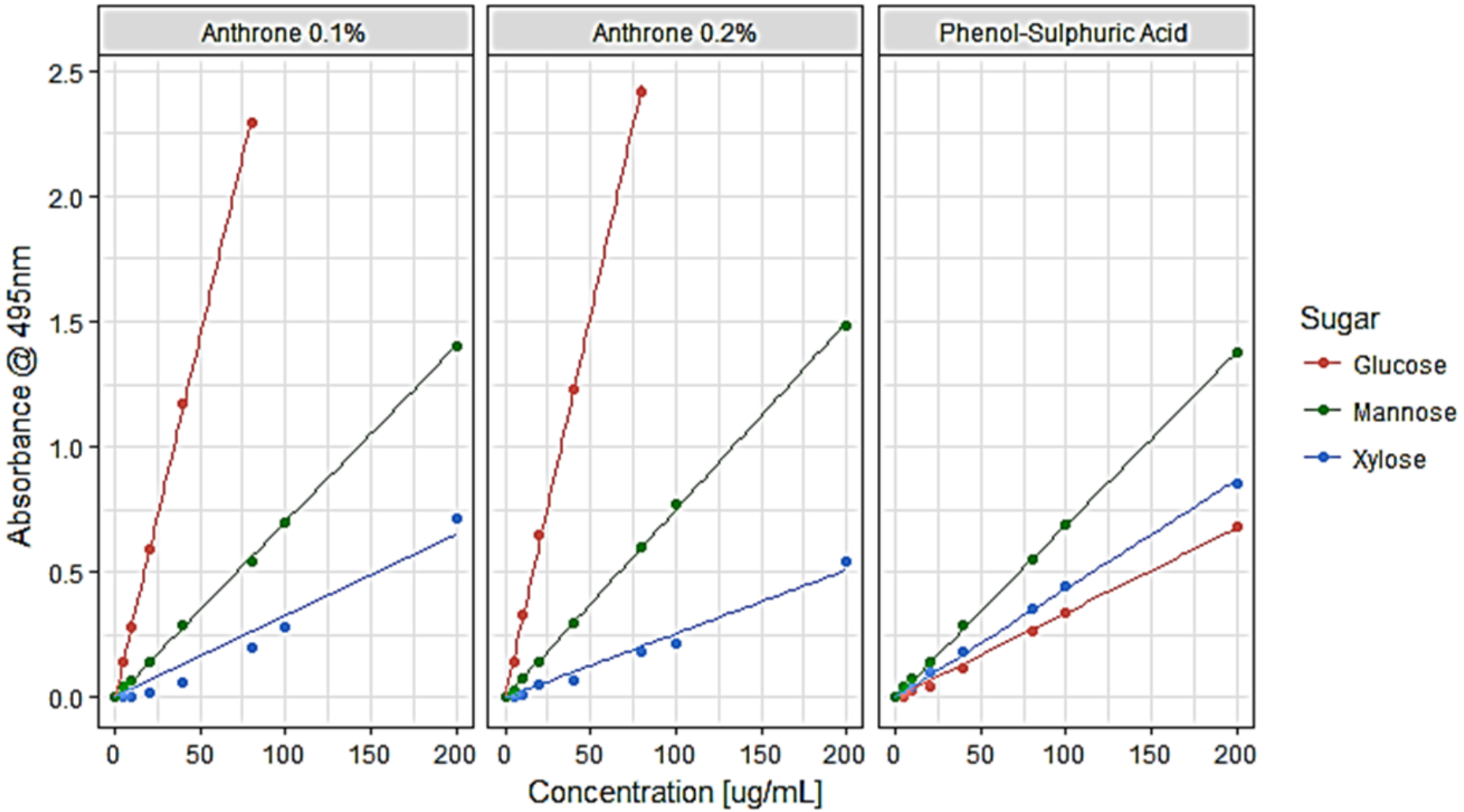


Fig.II-2 Comparison of methods for sugars determination

APPENDIX III: PROTEINS

Different assays can be used to estimate protein concentration in solution, among these the most commonly used are the spectrophotometric methods developed by Lowry in 1951 and Marion Bradford in 1976. Despite these methods provide relative measurements at best, it is common practice to quantify proteins from such data (Berges *et al.,* 1993). The most commonly used protein standard for calibration curves is Bovine Serum Albumin (BSA), but many others could be used. It is suggested that the Bradford and Lowry methods give different measurements when using BSA as standard for samples like higher plants and algae. To get more reliable measurements it would be useful to first identify the major proteins in the cells. However, this is practically unfeasible due to difficulties in extraction, purification and characterisation of the main proteins in the cells (Barbarino and Lourenço 2005)

III-I BRADFORD METHOD

The Bradford assay is relatively easy to perform and is based on the observation that the absorbance maximum for an acidic solution of the dye Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when it binds to proteins. Interactions are mainly with arginine rather than primary amino groups while the other basic (His, Lys) and aromatic residues (Try, Tyr, and Phe) give slight responses (Compton and Jones, 1985). Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change which is proportional to the amount of proteins in the sample.

This assay is very sensitive but is dependent on the quality of the protein (Sapan and Lundblad, 2015). A major disadvantage of this method derives from its variation in response to different proteins caused by the specificity of the assay towards arginine residues. Also,

for any given protein further discrepancies may arise from non-protein interferences that result in protein overestimation, underestimation and/or a reduction of the linear response range (Compton and Jones, 1985). The assay is linear over a short range therefore sample dilution before analysis are often necessary.

All chemicals were purchased from Sigma-Aldrich and solutions prepared in HPLC grade water. One mg/mL stock solutions of BSA or Lysozyme were freshly prepared and used to build calibration curves over the range 0-10 µg/mL for the Bradford assay and 0-100 µg/mL for the Lowry assay. For reactions, one ml of sample/standards was mixed with one ml of Bradford reagent and incubated at room temperature for 15 minutes. Sample/standards were then transferred to cuvettes and absorbance measure taken at a wavelength of 595 nm with a Jenway 7315 spectrophotometer using the 0 µg/ml standard as blank.

III-II LOWRY METHOD

Lowry assay is performed in two distinct steps. Protein is initially reacted with cupric sulphate at alkaline pH in the presence of tartrate for 10 minutes at room temperature. During this incubation, known as biuret reaction, a tetradentate copper complex is formed (Fig. III-1)

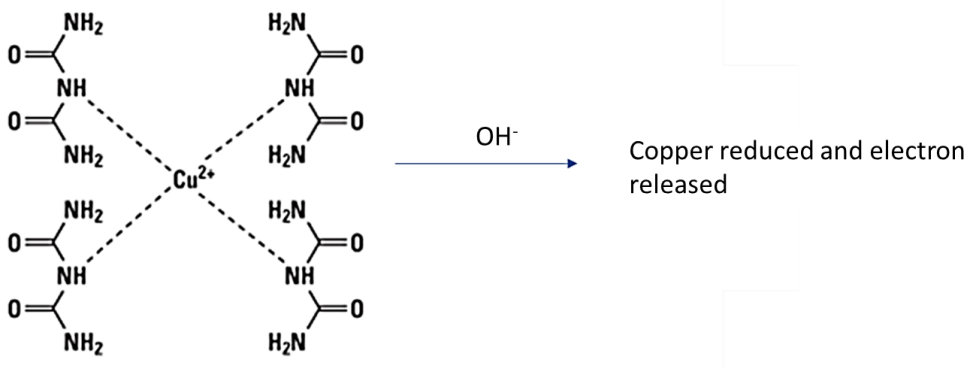


Fig.III-1 Biuret Reaction

A phosphomolybdic-phosphotungstic acid solution, known as Folin-phenol reagent, is then added. This is reduced, producing an intense blue colour which keeps intensifying during a 30-minute room temperature incubation. Full description of the method is given in Paragraph 4.3.3. It has been suggested that during this interval a rearrangement of the initial unstable blue complex leads to the stable final blue coloured complex which has higher absorbance (Lowry, et al. 1951; Legler, et al. 1985) and is optimally measured at 750nm. To maximise assay performance and allow the quantification of very dilute proteins solutions, samples preparation requires the removal of impurities and contaminants through quantitative precipitation using trichloro-acetic acid (TCA). Deoxycholate is also used to permit precipitation of proteins at low protein concentration (5-20µg/ml) (Sapan and Lundblad 2015). Also, detergents like sodium dodecyl sulfate (SDS) are often present in protein preparations to facilitate membranes solubilisation or removal of interfering substances. The sensitivity of this assay is moderately constant from protein to protein and it has been so widely used that estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures are involved (Waterborg and Matthews, 1984). The Lowry assay proved to be more reliable (R^2 = 0.9977 against R^2=0.9319 for Bradford assay), with a good linearity range (Fig III-2) and therefore selected for further analyses with BSA as standard.

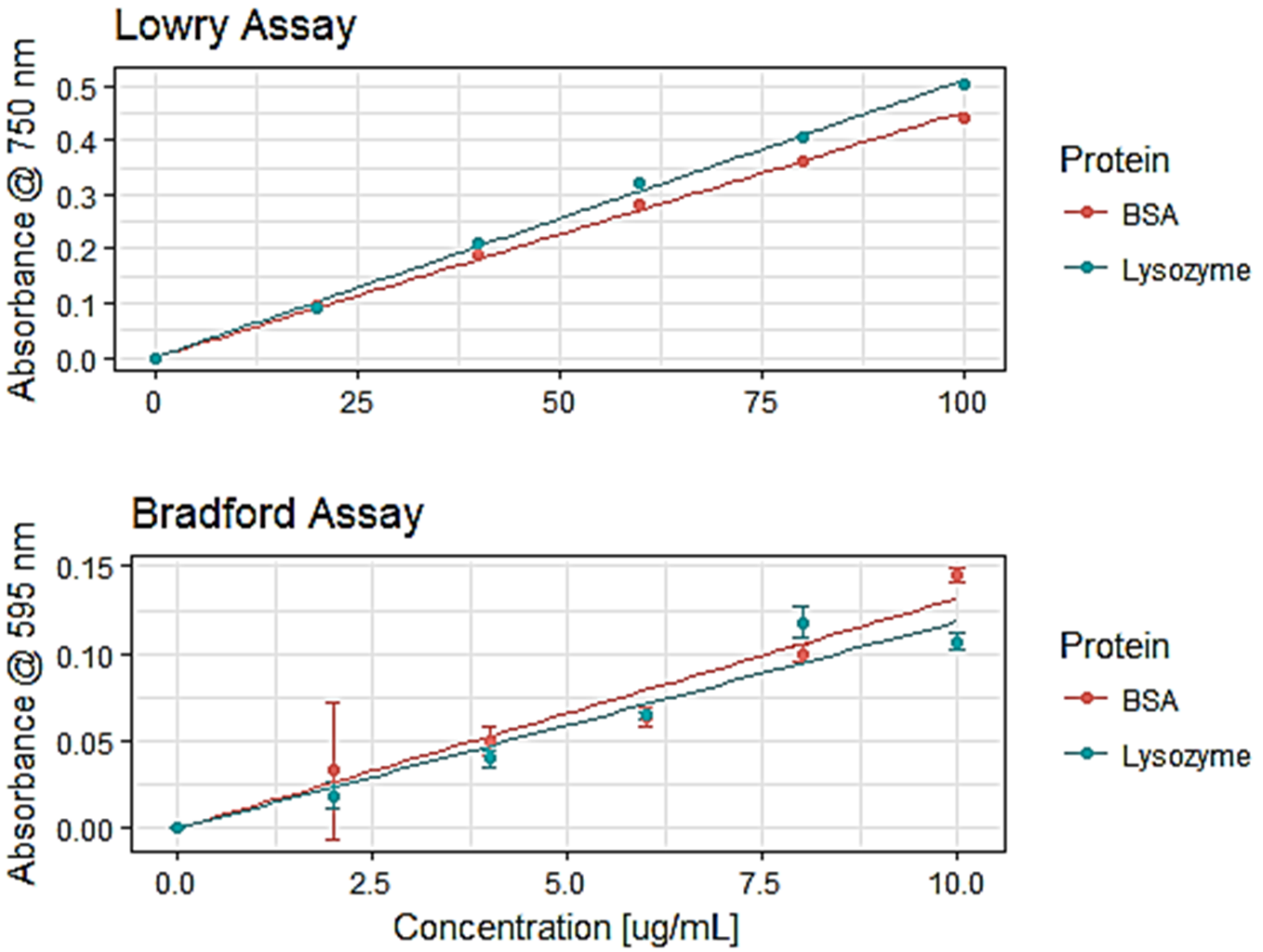


Fig.III-2 Comparison of protein assays

APPENDIX IV: URONIC ACIDS

Uronic acids are a class of sugars in which the hydroxyl group on the terminal carbon has been oxidised to carboxylic. It is reported that in algal and cyanobacterial EPS the presence of uronic acids confer a sticky character to the exudates macromolecules (Rossi and De Philippis, 2014) and in some cases responsible for flocculation of cells (Khangembam *et al.,* 2016).

IV-I THE ASSAY

Carbazole method was first introduced by Dische in 1946 for the quantitative spectrophotometric determination of uronic acids in biological samples. It was based on the principle that hexuronic acids treated with concentrated sulfuric acid highly specific produce mixtures of products which can react with carbazole to develop colours (Dische, 1946). A major disadvantage was however represented by the long time required for the full colour development (2h), which was also partially supressed by salts or other impurities in the reagents or samples (Bitter and Muir, 1962). Replacement of carbazole with meta-hydroxydiphenyl (Fig. IV-1) greatly improved the quantitative determination of uronic acids by reduction of the browning that occurs due to heat production in the acid hydrolysis step and avoiding the formation of additional interference by the carbazole reagent itself.

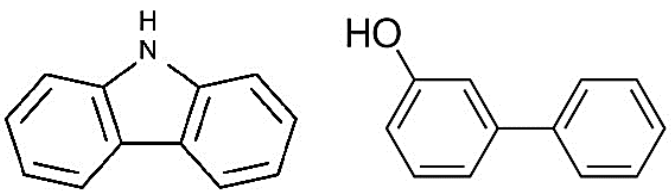


Fig.IV-1 Carbazole (left) and m-hydroxy-diphenyl reagent (right)

However, a major interference due to browning might occur during hydrolysis with sulfuric acid and before addition of the diphenyl reagent when uronic acids are determined in the presence of excess neutral sugar. This can be avoided by addition of sulfamate to the reaction mixture (Filisetti-Cozzi and Carpita, 1991). Full description of the method is given in Paragraph 4.3.4. The use of D-glucuronic acid as standard gave a good linear response over the range under study (R^2= 0.9804; 0-20 µg/mL) (Fig. 4-7) and therefore selected for further analyses.

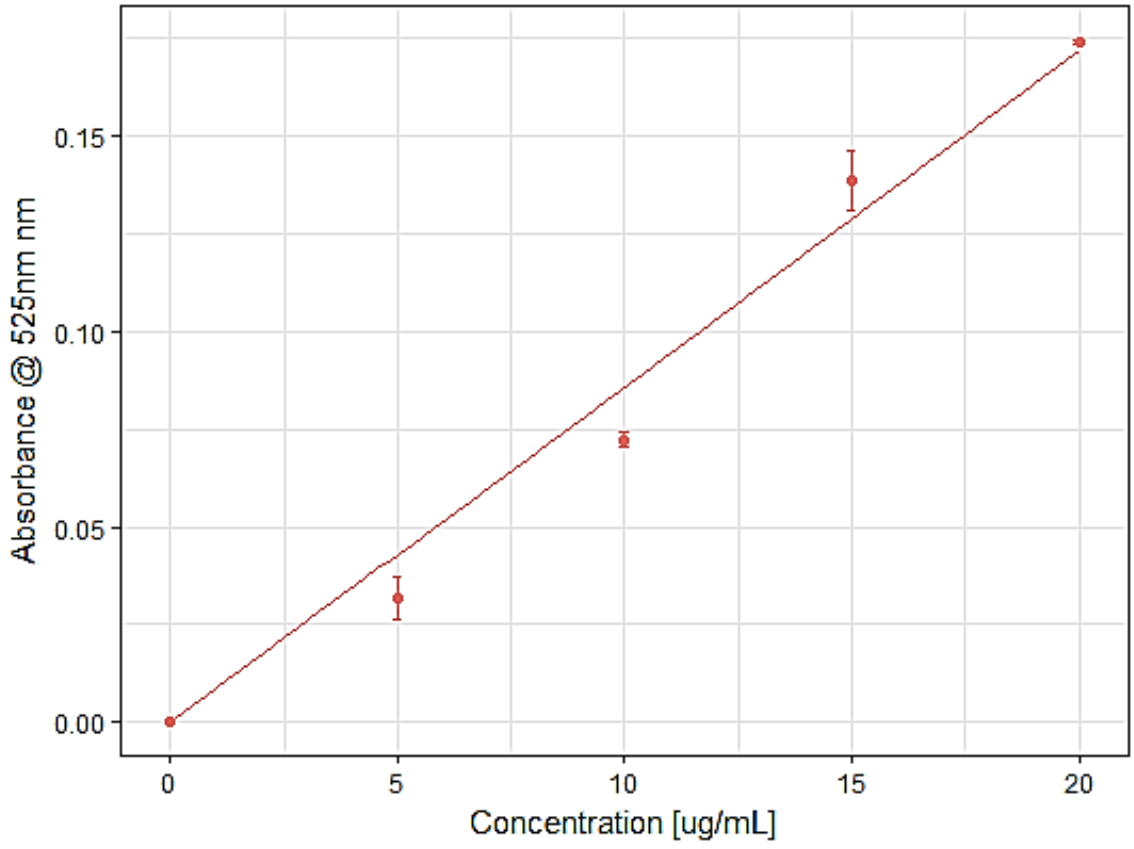


Fig.4-7 D-Glucuronic acid calibration curve

REFERENCES- CHAPTER IV

1. Al-Halbouni D, Dott W, Hollender J. Occurrence and composition of extracellular lipids and polysaccharides in a full-scale membrane bioreactor. Water Research. 2009;43(1):97-106.

2. Badireddy AR, Chellam S, Gassman PL, Engelhard MH, Lea AS, Rosso KM. Role of extracellular polymeric substances in bioflocculation of activated sludge microorganisms under glucose-controlled conditions. Water Research. 2010;44(15):4505-16.

3. Bailey RW. Reaction of pentoses with Anthrone. Biochemical Journal. 1958; 68:669-72.

4. Barbarino E, Lourenco SO. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. Journal of Applied Phycology. 2005;17(5):447-60.

5. Benemann JRaO, W. J. Systems and economic analysis of microalgae ponds for conversion of CO {sub 2} to biomass. Final report (No. DOE/PC/93204--T5). California Univ., Berkeley, CA (United States). Dept. of Civil Engineering; 1996.

6. Berges JA, Fisher AE, Harrison PJ. A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from the marine diatom Thalassiosira pseudonana. Marine Biology. 1993;115(2):187-93.

7. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Analytical Biochemistry. 1962;4(4):330-&.

8. Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry. 1976;72(1-2):248-54.

9. Compton SJ, Jones CG. Mechanism of dye response and interference in the Bradford protein assay. Analytical Biochemistry. 1985;151(2):369-74.

10. Cullimore DR. Practical Manual of Groundwater Microbiology, Second Edition: CRC Press; 2007.

11. Dische Z. A new specific color reaction of hexuronic acids. Journal of Biological Chemistry. 1947;167(1):189-98.

12. Dreywood R. Qualitative test for carbohydrate material. Industrial and Engineering Chemistry-Analytical Edition. 1946;18(8):499-.

13. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 1956;28(3):350-6.

14. D'Hondt E, Martin-Juarez J, Bolado S, Kasperoviciene J, Koreiviene J, Sulcius S, et al. Cell disruption technologies. Microalgae-Based Biofuels and Bioproducts2018. p. 133-54.

15. Filisetticozzi T, Carpita NC. Measurement of uronic-acids without interference from neutral sugars. Analytical Biochemistry. 1991;197(1):157-62.

16. Flemming H-C, Wingender J. The biofilm matrix. Nature Reviews Microbiology. 2010;8(9):623-33.

17. Guo S-L, Zhao X-Q, Wan C, Huang Z-Y, Yang Y-L, Alam MA, et al. Characterization of flocculating agent from the self-flocculating microalga Scenedesmus obliquus AS-6-1 for efficient biomass harvest. Bioresource Technology. 2013; 145:285-9.

18. Guo X, Wang X, Liu J. Composition analysis of fractions of extracellular polymeric substances from an activated sludge culture and identification of dominant forces affecting microbial aggregation. Scientific Reports. 2016;6: 28391

19. Harke MJ, Jankowiak JG, Morrell BK, Gobler CJ. Transcriptomic Responses in the Bloom-Forming Cyanobacterium Microcystis Induced during Exposure to Zooplankton. Applied and Environmental Microbiology. 2017;85(4): E02832-16.

20. Hessen DO, Vandonk E. Morphological changes in Scenedesmus induced by substances released from Daphnia. Archiv Fur Hydrobiologie. 1993;127(2):129-40.

21. Jakob G, Stephens E, Feller R, Oey M, Hankamer B, Ross IL. Triggered exocytosis of the protozoan Tetrahymena as a source of bioflocculation and a controllable dewatering method for efficient harvest of microalgal cultures. Algal Research-Biomass Biofuels and Bioproducts. 2016; 13:148-58.

22. Juneja A, Ceballos RM, Murthy GS. Effects of Environmental Factors and Nutrient Availability on the Biochemical Composition of Algae for Biofuels Production: A Review. Energies. 2013;6(9):4607-38.

23. Khangembam R, Tiwari ON, Kalita MC. Production of exopolysaccharides by the

cyanobacterium Anabaena sp BTA992 and application as bioflocculants. Journal of Applied Biology & Biotechnology. 2016;4(1):8-11.

24. Lampert W, Rothhaupt KO, Vonelert E. Chemical Induction of colony formation in a green-alga (Scenedesmus acutus) by grazers (Daphnia). Limnology and Oceanography. 1994;39(7):1543-50.

25. Le C, Stuckey DC. Colorimetric measurement of carbohydrates in biological wastewater treatment systems: A critical evaluation. Water Research. 2016; 94:280-7.

26. Legler G, Mullerplatz CM, Mentgeshettkamp M, Pflieger G, Julich E. On the chemical basis of the Lowry protein determination. Analytical Biochemistry. 1985;150(2):278-87.

27. Li M, Zhu W, Dai X, Li X. Effects of linear alkylbenzene sulfonate on extracellular polysaccharide content and cells per particle of Microcystis aeruginosa and Scenedesmus obliquus. Fresenius Environmental Bulletin. 2013;22(4B):1189-94.

28. Li XY, Yang SF. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Research. 2007;41(5):1022-30.

29. Liang Z, Li W, Yang S, Du P. Extraction and structural characteristics of extracellular polymeric substances (EPS), pellets in autotrophic nitrifying biofilm and activated sludge. Chemosphere. 2010;81(5):626-32.

30. Liu H, Fang HHP. Extraction of extracellular polymeric substances (EPS) of sludges.

Journal of Biotechnology. 2002;95(3):249-56.

31. Liu Y, Wang W, Zhang M, Xing P, Yang Z. PSII-efficiency, polysaccharide production, and phenotypic plasticity of Scenedesmus obliquus in response to changes in metabolic carbon flux. Biochemical Systematics and Ecology. 2010;38(3):292-9.

32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951;193(1):265-75.

33. Lurling M. Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. Annales De Limnologie-International Journal of Limnology. 2003;39(2):85-101.

34. Lürling M. The smell of water. Grazer-induced colony formation in Scenedesmus.: Agricultural University of Wageningen; 1999.

35. Lürling M. Grazer-induced coenobial formation in clonal cultures of Scenedesmus obliquus (Chlorococcales, Chlorophyceae). Journal of Plankton Research. 1999A; 23:19-23.

36. Manheim D, Nelson Y. Settling and Bioflocculation of Two Species of Algae Used in Wastewater Treatment and Algae Biomass Production. Environmental Progress & Sustainable Energy. 2013;32(4):946-54.

37. Maqbool T, Khan SJ, Waheed H, Lee C-H, Hashmi I, Iqbal H. Membrane biofouling retardation and improved sludge characteristics using quorum quenching bacteria in submerged membrane bioreactor. Journal of Membrane Science. 2015; 483:75-93.

38. Milledge JJ, Heaven S. A review of the harvesting of micro-algae for biofuel

production. Reviews in Environmental Science and Bio-Technology. 2013;12(2):165-78.

39. Mishra A, Jha B. Isolation and characterization of extracellular polymeric substances from micro-algae Dunaliella salina under salt stress. Bioresource Technology. 2009;100(13):3382-6.

40. More TT, Yan S, John RP, Tyagi RD, Surampalli RY. Biochemical diversity of the bacterial strains and their biopolymer producing capabilities in wastewater sludge. Bioresource Technology. 2012; 121:304-11.

41. O'Donnell DR, Fey SB, Cottingham KL. Nutrient availability influences kairomone-induced defenses in Scenedesmus acutus (Chlorophyceae). Journal of Plankton Research. 2013;35(1):191-200.

42. Phuc-Nguon H, Honda R, Noguchi M, Ito T. Optimum selection of extraction methods of extracellular polymeric substances in activated sludge for effective extraction of the target components. Biochemical Engineering Journal. 2017; 127:136-46.

43. Pickettheaps JD, Staehelin LA. Ultrastructure of Scenedesmus (Chlorophyceae). 2. Cell-division and colony formation. Journal of Phycology. 1975;11(2):186-202.

44. Pohnert G, Steinke M, Tollrian R. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends in Ecology & Evolution. 2007;22(4):198-204.

45. Quigg A, Passow U, Chin WC, Xu C, Doyle S, Bretherton L, et al. The role of microbial exopolymers in determining the fate of oil and chemical dispersants in the ocean.

Limnology and Oceanography Letters. 2016;1(1):3-26.

46. Rossi F, De Philippis R. Role of Cyanobacterial Exopolysaccharides in Phototrophic Biofilms and in Complex Microbial Mats. Life-Basel. 2015;5(2):1218-38.

47. Sapan CV, Lundblad RL. Review of methods for determination of total protein and peptide concentration in biological samples. Proteomics Clinical Applications. 2015;9(3-4):268-76.

48. Shen Y, Xu X, Zhao Y, Lin X. Influence of algae species, substrata and culture conditions on attached microalgal culture. Bioprocess and Biosystems Engineering. 2014;37(3):441-50.

49. Sheng G-P, Yu H-Q, Li X-Y. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances. 2010;28(6):882-94.

50. Takahashi E, Ledauphin J, Goux D, Orvain F. Optimising extraction of extracellular polymeric substances (EPS) from benthic diatoms: comparison of the efficiency of six EPS extraction methods. Marine and Freshwater Research. 2009;60(12):1201-10.

51. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

52. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology. 2013;31(4):233-9.

53. Wang Y-S, Tong Z-H, Wang L-F, Sheng G-P, Yu H-Q. Effective flocculation of Microcystis aeruginosa with simultaneous nutrient precipitation from hydrolyzed human urine. Chemosphere. 2017; 193:472-8.

54. Waterborg JH. The Lowry Method for Protein Quantitation. Protein Protocols Handbook, Third Edition. 2009:7-10.

55. Whitton BA, Potts M. The ecology of cyanobacteria: their diversity in time and space.: Springer Science & Business Media; 2007.

56. Wilen B-M, Lumley D, Mattsson A, Mino T. Relationship between floc composition and flocculation and settling properties studied at a full scale activated sludge plant. Water Research. 2008;42(16):4404-18.

57. Wingender J, Neu TR, Flemming HC. Microbial Extracellular Polymeric Substances: Characterisation, Structure and Function. Berlin: Springer; 1999.

58. Wu X, Zhang J, Qin B, Cui G, Yang Z. Grazer density-dependent response of induced colony formation of Scenedesmus obliquus to grazing-associated infochemicals. Biochemical Systematics and Ecology. 2013; 50:286-92.

59. Xiao R, Zheng Y. Overview of microalgal extracellular polymeric substances (EPS) and their applications. Biotechnology Advances. 2016;34(7):1225-44.

60. Xu H, Jiang H, Yu G, Yang L. Towards understanding the role of extracellular polymeric substances in cyanobacterial Microcystis aggregation and mucilaginous bloom formation. Chemosphere. 2014; 117:815-22.

61. Yang Z, Kong F. Formation of large colonies: a defense mechanism of Microcystis

aeruginosa under continuous grazing pressure by flagellate Ochromonas sp. Journal of Limnology. 2012;71(1):61-6.

62. Yang Z, Kong F, Shi X, Xing P, Zhang M. Effects of Daphnia-associated infochemicals on the morphology, polysaccharides content and PSII-Efficiency in Scenedesmus obliquus. International Review of Hydrobiology. 2007;92(6):618-+.

63. Yang Z, Liu Y, Ge J, Wang W, Chen Y, Montagnes D. Aggregate formation and polysaccharide content of Chlorella pyrenoidosa Chick (Chlorophyta) in response to simulated nutrient stress. Bioresource Technology. 2010;101(21):8336-41.

64. Yasumoto K, Nishigami A, Aoi H, Tsuchihashi C, Kasai F, Kusumi T, et al. Isolation of new aliphatic sulfates and sulfamate as the Daphnia kairomones inducing morphological change of a phytoplankton Scenedesmus gutwinskii. Chemical & Pharmaceutical Bulletin. 2008;56(1):133-6.

65. Zhang W, Cao B, Wang D, Ma T, Xia H, Yu D. Influence of wastewater sludge treatment using combined peroxyacetic acid oxidation and inorganic coagulants re-flocculation on characteristics of extracellular polymeric substances (EPS). Water Research. 2016; 88:728-39.

66. Zhong C, Xu A, Chen L, Yang X, Yang B, Hong W, et al. Production of a bioflocculant from chromotropic acid waste water and its application in steroid estrogen removal. Colloids and Surfaces B-Biointerfaces. 2014; 122:729-37.

67. Zhu W, Dai X, Li M. Relationship between extracellular polysaccharide (EPS) content and colony size of Microcystis is colonial morphology dependent. Biochemical

Systematics and Ecology. 2014; 55:346-50.

68. Zhu X, Nan H, Chen Q, Wu Z, Wu X, Huang Y, et al. Potential grazing intensity directly determines the extent of grazer-induced colony formation in Scenedesmus obliquus. Biochemical Systematics and Ecology. 2015; 61:271-7.

CHAPTER V

A Proteomic Investigation of Scenedesmus subspicatus flocculation in response to infochemicals

5.1 Introduction

Unravelling the molecular mechanisms allowing algae to flocculate and produce colonies is of major interest within the fields of ecology (Lürling 2003, van Holthoon *et al.,* 2003, Pohnert *et al.,* 2007, O'Donnell *et al.,* 2013, Wu *et al.,* 2013, Zhu *et al.,* 2015), evolution (Fischer et al., 2014) and engineering (Montemazzani et al., 2015, Alam et al., 2016, Roccuzzo et al., 2016, Zhu et al., 2017). For the latter, understanding how these natural cues trigger flocculation is particularly relevant to the large scale algal cultivation, where manipulating the formation of flocs is central to harvesting in an economically sustainable and "clean" manner. The current theory about flocculation centres on the production of EPS, thought to be a 'glue' that helps bind cells together (Wilen et al., 2008, Bogino et al., 2013, Lee et al., 2016).

This thesis (Chapter III and IV) and other research work (Yang et al., 2007, Yang and Kong, 2012, Harke et al., 2017) have documented and experimentally reported that *Scenedesmus* spp. and other microalgal/cyanobacterial species respond to grazing stress from *Daphnia spp*. producing EPS, forming colonies and flocculating. Despite being acknowledged as a defence mechanism, the leading cellular processes, the nature of EPS production and how we can exploit the molecular mechanisms behind it for biotechnology applications still needs to be fully disclosed.

To date, genomics, transcriptomics and proteomics approaches have been proposed and trialled to analyse pathways and functions linked to EPS production, flocculation and colony formation (Prochnik et al., 2010, Gulez et al., 2014, Schmidt et al., 2015, Yu et al., 2015, Khona et al., 2016, Harke et al., 2017). Here the focus is on the proteomic response of *S. subspicatus* to naturally occurring chemical cues from an herbivore grazer, *Daphnia magna*.

The overarching objective was to reveal major metabolic pathways (e.g. protein, lipid and carbohydrate synthesis, stress responses) altered by exposure to the cues and central to the formation of flocs and EPS. The approach used in this thesis relies on quantitative proteomics.

5.1.1 Why Proteomics?

The proteome is complex and variable under the effect of several stress factors. The study of the proteomes under a given stress can reveal metabolic changes directly as proteins include enzymes involved in metabolite level regulation as well as components of the transcription and translation machinery, therefore representing direct players in the stress response (Kosova' et al. 2011). Proteomics studies allow the determination of many properties, such as protein abundance, post-translational modifications (PTMs) and protein-protein interactions above all, therefore providing a comprehensive overview of the changes which occur during a certain biological process (Gonneaud et al., 2017).

Several examples can be found in the literature on how chemically mediated interactions alter phytoplankton metabolism and/or defence responses. Poulson-Ellestad et al. in 2014 reported a combined metabolomics and proteomics study where allelopathy, i.e. release of compounds that inhibit competitors, and which play an important part in the maintenance of large blooms of the dinoflagellate *Karenia brevis* as mono-specie against multiple diatoms competitors, showed to cause highly altered metabolic processes in diatoms, indicative of increased stress (e.g. oxidative stress), and cellular processes including photosynthesis, glycolysis and cell membrane restructuring (e.g. altered cell components) . Moreover, gel-like glycoproteins were more abundant in exposed diatoms exposed, suggesting a trigger for the aggregation of cells as a defense mechanism. Harke et al., in 2017 performed a

transcriptomic study of the response of the cyanobacterium *Microcystis* to direct and indirect *exposure* to *Daphnia* grazers, reporting a higher transcription of genes related to secondary metabolites with putative role in defense against grazing (e.g., microcystin peptide synthesis genes), heat shock proteins as well as photosynthetic processes, indicating a *Daphnia* induced stimulation of energy acquisition pathways. Also, gene transcripts associated with production and export of sugar-EPS (i.e. tagH, rfbB, rfbC and rfbD) were significantly increased upon exposure to infochemicals and linked to colony formation of *Microcystis* as a defense against grazing (Harke et al., 2017).

This chapter will show how the proteome of the microalga *S. subspicatus* responds to infochemicals from its *D. magna* water flea grazer. Several classes of proteomic responses are expected to be observed, including energy, lipids and carbohydrates metabolism, photosynthesis and proteins synthesis/degradation. In fact, in the case flocculation is driven by EPS production, this should be reflected by metabolisms costs related to the supply of EPS precursors. Regarding colony formation and therefore a pathway where the division of a single mother cell leads the daughter cells to stay connected by a common cell wall (Bisova' et al., 2013), it is expected to observe variation in regulation of proteins involved in cell cycle and division (Li et al., 2016, Pillai et al., 2014, Wei et al., 2017).

Scenedesmus spp have attractive features for industrial applications; however, they do not represent model-organisms in molecular research and the use of proteomics to unravel the infochemicals response in *S. subspicatus* required to match the spectra to the proteomes of a series of closely-related organisms (Carpentier et al., 2008, Armengaud et al., 2014). For un-sequenced organisms, an alternative to this procedure would be represented by *de-novo* sequencing, where the mass difference between two fragment ions observed in MS/MS is

used to compute the mass of an amino acid residue. However, not every ion from the theoretical fragmentation are observed in MS/MS spectra, counterfeit assignments might be produced or many peaks missed (Allmer, 2011). Results are here presented from a replicated experiment revealing patterns of altered protein expression in these major pathways, using iTRAQ in a shot-gun proteomics approach. These data provide a platform for developing a better understanding of colony formation and flocculation in microalgae, paving the way for application in algal biotechnology for small and large scale, economically viable harvesting of algal biomass.

5.2 METHODS

In this thesis, the main goal was to study the impact of *Daphnia* infochemicals as the cause of flocculation and colony formation in *S. subpsicatus*. To do so, the effects caused by the infochemicals carrier (the salty medium ASTM, required by *Daphnia* to live) were distinguished by those caused by infochemicals (ASTM+ *Daphnia* cues) and both compared to non-stressed conditions of *Scenedesmus* proteome. Changes were observed at early exponential stage of algal cells and for two-time points of exposure: +2 and +20 hours. These were chosen to observe variations early enough under infochemicals effects and at a time after which no further flocculation is observed (Chapter III). Two fractions were collected for *S. subspicatus* cultures exposed to infochemicals: the lower part- *flocs*, and the upper part – *planktonic cells*. In fact, it was previously mentioned in Chapter II that a distinction between colony formation and aggregation-based mechanisms is necessary (Table 5-1).

Table 5-1 *Phenotypes comparisons and related biological motivations*

TIME POINT	PHENOTYPES COMPARISON	MOTIVATION	
+2H	ASTM vs. CONTROL	Changes due to the presence of salts in the *Daphnia* culturing medium – "carrier effect"	Changes caused at the alarm phase - upon early detection of cues
	DW $_{PLK}$ vs. CONTROL/ASTM	Changes due to infochemicals – colony formation	
	DW F_{LOC} vs. CONTROL/ASTM	Changes due to infochemicals - flocculation	
	DW $_{FLOC}$ vs. DW $_{PLK}$	Colony formation vs. flocculation	
+20H	ASTM vs. CONTROL	Changes due to the presence of salts in the *Daphnia* culturing medium – "carrier effect"	Changes caused at the acclimation phase – after which no increase of flocculation efficiency is observed
	DW $_{PLK}$ vs. CONTROL/ASTM	Changes due to infochemicals – colony formation	
	DW $_{FLOC}$ vs. CONTROL/ASTM	Changes due to infochemicals - flocculation	
	DW $_{FLOC}$ vs. DW $_{PLK}$	Colony formation vs. flocculation	

Among the available techniques for quantitative proteomics, iTRAQ (isobaric tags for relative and absolute quantitation) was chosen to perform this experimental work, as it is a well-established chemical labelling method in quantitative proteomics for microalgae (Longworth et al., 2016, Shi et al., 2017, Helliwell et al., 2017). Based on the labelling of the N-terminus of peptides generated after enzymatic digestion, it can be used for a wide range of biological samples and represents a robust technique, with multiple conditions compared in one experiment (Evans et al., 2012).

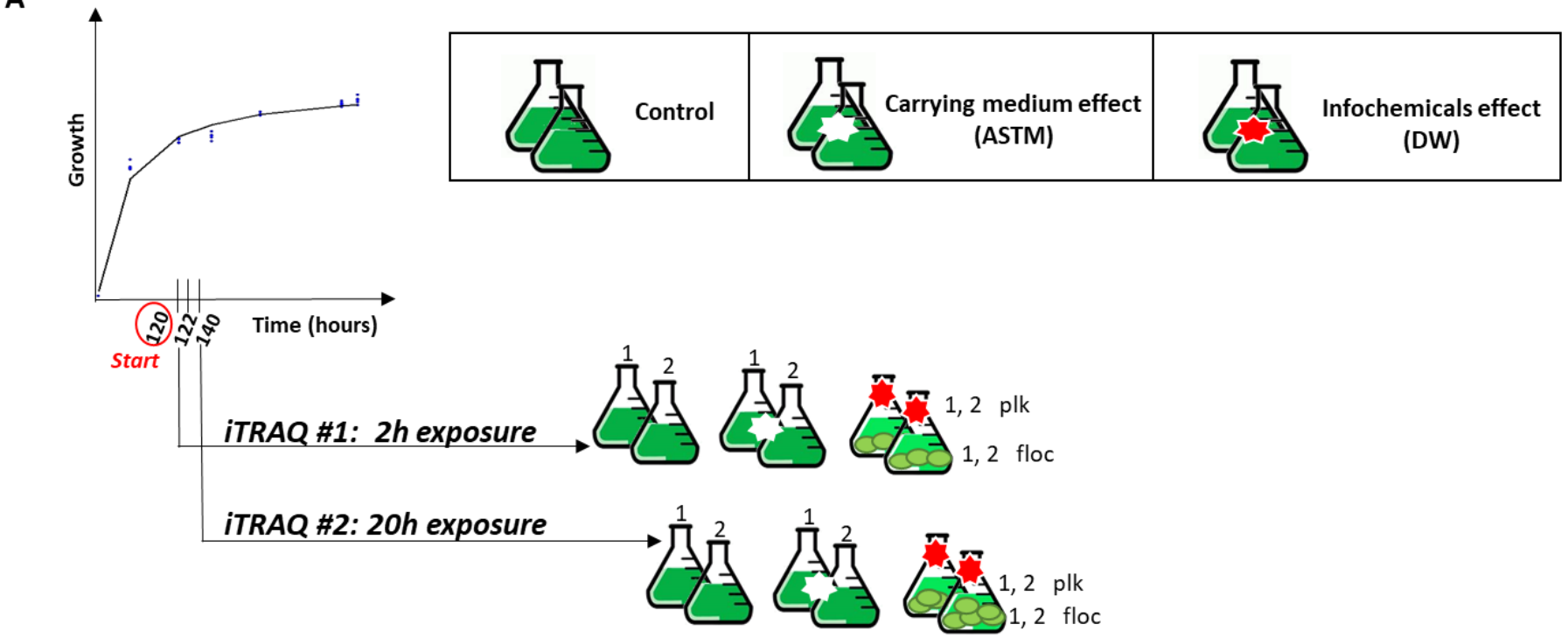
5.2.1 Culture Conditions and Infochemicals Production

S. subspicatus (strain NIVA-CHL 97) was maintained in the lab in Ebert's medium (composition described in Chapter III, Paragraph 3.2.1) and cultured in 250 mL Erlenmeyer flasks at 20 ± 1°C, continuously illuminated under light at 259 µmol/ m^2·s. *Daphnia magna* used to produce the infochemicals was a laboratory clone maintained in the lab for several months in a temperature controlled room at 20 ± 1°C in a 16:8 light-dark cycle, cultured in one L jars with ASTM hard water and fed daily with 250 µL of *S. subspicatus* cells ($2 \cdot 10^5$ cells/mL). To produce the infochemicals, animals were incubated at a density of 100 ind/L with *S. subspicatus* as food. Animals were removed after 24 hours and the culture filtered through a 0.2 µm cellulose acetate filter (Sartorius Stedim Biotech Gmbh, Germany) to obtain the *Daphnia* test water (DW).

5.2.2 Experimental Design

Five mL of exponentially growing *S. subspicatus* (~10^6 cells/mL) were transferred to 250 mL Erlenmeyer flasks containing 150 mL of autoclaved Ebert's medium and let grow until early exponential stage; at this point either five mL of additional culture medium – Control - or five mL of DW or five mL of ASTM water were added to the biological replicates (n=2). Batch cultures were incubated at 20±1°C on a shaking table at 120 rpm, continuously illuminated from above by light tubes at 259 µmol/m2·s and randomly rearranged daily. Sampling was performed after +2h and +20h of exposure. Experimental design and preparation of cultures for proteome analysis are outlined in Figure 5-1 Panel A and B, respectively.

A



Time of exposure	Treatment	iTRAQ label
+2h (iTRAQ#1)	Control (#1)	113
	Control (#2)	114
	ASTM (#1)	115
	ASTM (#2)	116
	DWplk (#1)	117
	DWplk (#2)	118
	DWfloc (#1)	119
	DWfloc (#2)	121

Time of exposure	Treatment	iTRAQ label
+20h (iTRAQ#2)	Control (#1)	113
	Control (#2)	114
	ASTM (#1)	115
	ASTM (#2)	116
	DWplk (#1)	117
	DWplk (#2)	118
	DWfloc (#1)	119
	DWfloc (#2)	121

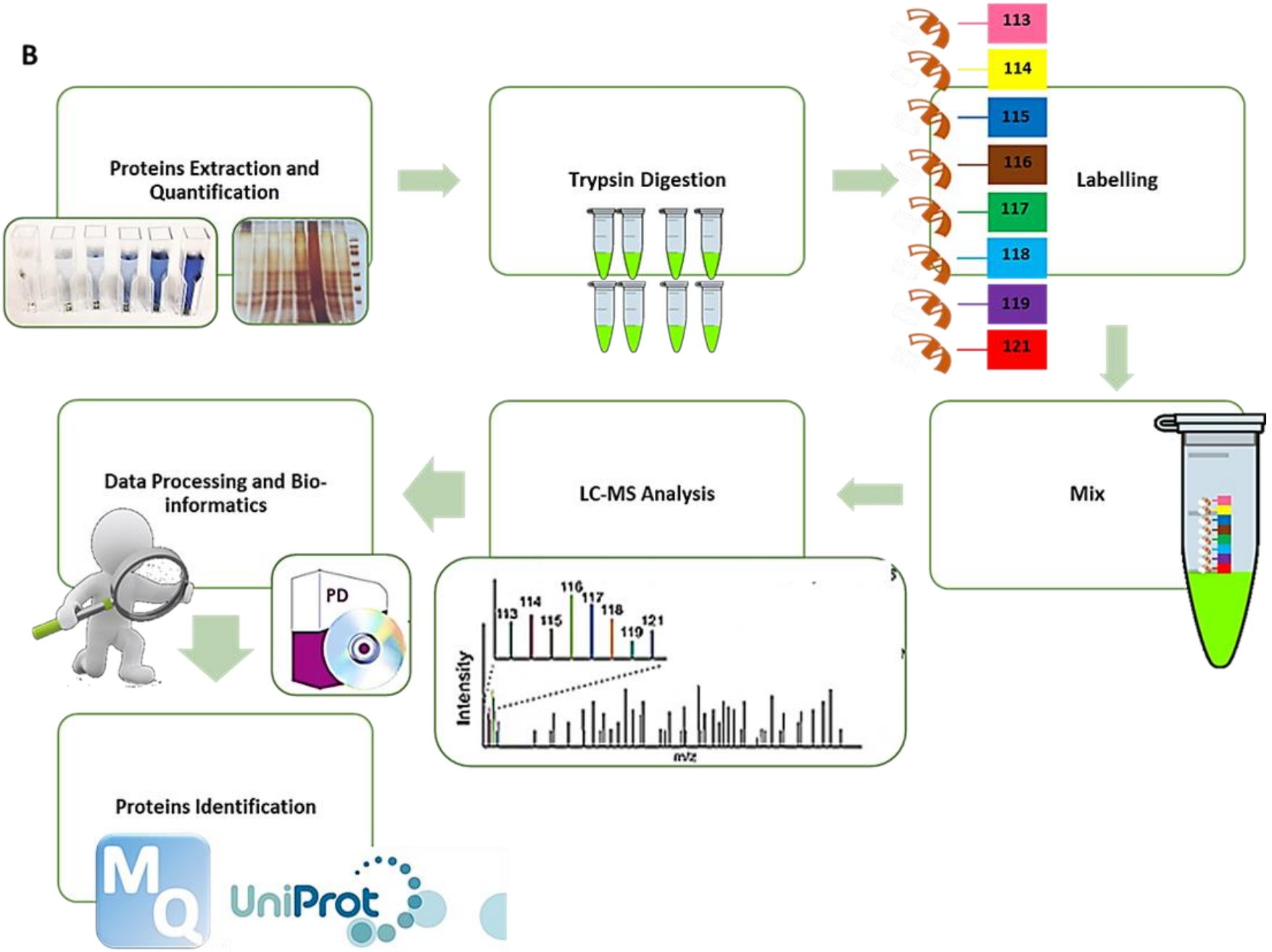


Fig. 5-2 A schematic representation of the experimental design (A) and proteomics workflow (B)

5.2.3 Protein Preparation & Quantification

Algal cells were harvested after five days plus either 2h or 20h of exposure to either ASTM or DW, and then pelleted by centrifugation at 3000 g for 15 min at 4 °C. Cultures exposed to DW exhibited flocculation and supernatant (planktonic) fraction was separated from the floc fraction. Algal cell pellets were washed with triethylammonium bicarbonate buffer (TEAB), transferred to a protein low bind tube and centrifuged again at 3000 g for 10 min. Pellets were then resuspended in 250 µL of lysis buffer composed by 200 mM TEAB, 10mM DL-dithiothreitol (DTT), 0.5% sodium deoxycholate and enzymatic protease inhibitor. With the use of buffers, detergents, salts and reducing agents cell are lysed and proteins are solubilised; protease inhibitors protect the extracted proteins from degradation or modification by the activities of these enzymes. Reagent based lysis was followed by physical lysis in the form of a combination of liquid Nitrogen (LN_2) cracking and bead-beating. A pestle and mortar, wiped with 70% ethanol was pre-chilled using LN_2. More LN_2 was subsequently poured and the samples ground with a pestle for 10 min each. This step was repeated three times in total and the samples were finally collected with a spatula and transferred in a Lo-Bind tube. Sample underwent bead-beating, using 100 µg of zirconia beads and a cell disruptor, with five cycles of alternative one-minute beating and one-minute incubation on ice. Unbroken cells and cell debris were pelleted by centrifugation at 18,000 g for five minutes and the supernatants transferred to clean Lo-Bind tubes. The total protein concentration was estimated by the Lowry's method (described in Chapter IV-EPS).

5.2.4 Proteins Digestion and Labelling

Aliquots of samples containing 100 µg protein was added with five µL of 10 mM Tris-(2-carboxyethyl)-phosphine (TCEP) for reduction and incubated at 60 °C for 30 min. Then, samples were alkylated by adding 6 µL of 200 mM methyl methane-thiosulfonate (MMTS) in

Dimethyl sulfoxide (DMSO), and incubated for 30 minutes at room temperature. Samples were then digested with 1:20 trypsin in TEAB and incubated overnight at 37 °C. iTRAQ labelling reagents were removed from the freezer immediately prior to use, brought to room temperature, spun in a microfuge at 3000 g for one minute and resuspended in 50 µL of isopropanol. Labelling reagents were vortexed well, centrifuged again, and the whole vials content added to the samples. These were then incubated at room temperature for two hours, combined in one tube and finally dried overnight in a vacuum centrifuge at 30 °C. Sample was then resuspended in 100 µL Hyper-carb buffer (3% Acetonitrile ACN + 0.1% trichloro acetic acid – TCA), ready for fractionation.

5.2. 5 HPLC Procedure

HPLC was performed using an Hypercarb™ column, which is packed with pH-stable carbon particles (porous graphitic carbon- PGC) and allow the separation of biomolecules on the basis of their hydrophobicity and molecular geometry (Pereira, L., 2010). Two buffer solutions were prepared: a) *Buffer A*: 3% ACN + 0.1% TFA and b) *Buffer B*: 97% ACN and 0.1% TFA. The Hypercarb™ separation performed on a Dionex UltiMate 3000 Autosampler linked to Dionex UltiMate 3000 Flow Manager and Pump system (Thermo Scientific, UK). Samples were re-suspended in 200 µL *Buffer A* and loaded onto Hypercarb™ Porous Graphitic Carbon LC reversed phase Analytical Column (Cat no. 35003-052130, ThermoFisher Scientific, UK), with 3 µm particle size, 50 mm length, 2.1 mm diameter and 250 Å pore size. *Buffer A* was exchanged with *Buffer B* with a flow rate of 30 µL/min with the following gradient: 3% B at 0-10 minutes, 10% B at 10-85 minutes, 50% B at 85-86 minutes, 90% B at 86-91inutes, 3% B at 91-105 minutes. The fractions were collected every two minutes from 20 to 120 minutes. The fractions were dried for 20 hours on a Scanvac vacuum centrifuge (Labogene, Denmark,

Serial no. GVS23511110026) connected to a Vacuubrand Vacuum Pump (Vacuubrand, Germany) ready for recombination and mass spectrometry analysis.

5.2.6 Mass Spectrometry

AmaZon ETD MS was used in CID (collision induced dissociation) mode to test a small aliquot of digested proteins to check for miscleavages and incomplete digestion. AmaZon ion-trap ETD MS was connected to Dionex UltiMate 3000 Autosampler linked to Dionex UltiMate 3000 Flow Manager and Pump system (Thermo Scientific, UK). Chromeleon software was used to control the loading and running of samples, and recording of data. Data was analysed using DataAnalysis software and searched in Mascot. LC-MS/MS was then performed and analysed by nano-flow liquid chromatography (U3000 RSLCnano, Thermo Scientific) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive HF, Thermo Scientific). iTRAQ-peptides were separated on an Easy-Spray C18 column (75 µm x 50 cm) using a 2-step gradient from 97% solvent A (0.1% formic acid -FA -in water) to 10% solvent B (0.08% FA in 80% ACN) over five min then 10% to 50% B over 75 min at 300 nL/min. The mass spectrometer was programmed for data dependent acquisition with the following settings: resolution 30,000, automatic gain control (AGC) target 1e5, maximum injection time 60ms, isolation window 2.0 m/z, normalised collision energy 27, intensity threshold 3.3e4, per full MS scan (resolution 120,000, AGC 1e6, maximum injection time 60ms, scan range 375 to 1500 m/z, polarity positive).

5.2.7 Fatty Acids

Five mL of algal cultures were transferred to 15 mL falcon tubes and centrifuged at 8000 rpm for three minutes. Four mL of supernatant (media) were decanted and the pellets re-suspended in the remaining one mL leftover media. Cell suspensions were then transferred

to pre-weighed 2 mL Eppendorf tubes. The algal suspensions were centrifuged for two minutes at 13000 rpm and at 4°C. The supernatants were discarded and the Eppendorf tubes weighed to estimate the wet algal biomass. Samples (three biological replicates per treatment and three technical replicates each) were sent to another laboratory for determination of fatty acids via direct transesterification followed by gas chromatography analysis (courtesy of Dr R. Kapoore, University of Sheffield).

5.3 Data Analysis

The fraction files were processed in data analysis software MaxQuant, the standard software for processing Q Exactive HF MS data (Michalski et al., 2011). The data were searched against a customised proteome database (UniProt IDs) comprehensive of green algae and cyanobacteria data with a total of 97,523 entries (downloaded on June 2017). Searches were carried using the following settings: Enzyme: Trypsin; Fixed PTMs: β-methylthio (MMTS); Variable PTMs: Oxidation [M], Deamidation [NQ], iTRAQ [Y]; labelling: iTRAQ 8-plex; max miscleavages: 3; false discovery rate (FDR): 1%; min number of unique peptides: 1. MaxQuant employs a sequence database search to find the best peptide match explaining the observed peaks in the MS/MS spectrum (Zhang et al., 2012). The lists of peptides generated were then used to compute relative quantifications of proteins using in-house software uTRAQ (Application creator: J. Noirel, 2013)

5.3.1 Replicates Consistency

PCA on protein abundance is a common method to visualise high dimension data and reveal major groups of proteins that are correlated and independent of other groups (Baumann et al., 2010, Alonso-Gutierrez et al., 2015). It is commonly used in proteomics (Yang et al.,

2015, Shi et al., 2017) to reveal whether biological replicates share similar patterns and whether biological treatments are differentiated.

In the present work, PCA was applied to the isotope and median corrected (IC, MC) peptide intensities (see supporting material section II) to first check on biological groupings and second to formally test whether the treatments are significantly different with respect to the PCA axes, using a permutation based analysis of variance (Adonis method). The major axes returned by the PCA also offer a first insight into proteins linked, via abundance, to different treatments. We used the *rda* and *adonis* functions from the R package *vegan* for the PCA and visualisation of data (Oksanen, 2017).

5.3.2 Phenotypes Comparison and Analysis of Differentially Expressed Proteins (DEPs)

Differential expression of proteins was analysed using the University of Sheffield in-house programs *uTRAQ* and *SignifiQuant* (Applications creator: J. Noirel, 2013). *uTRAQ* is a program which uses a peptide spectral match (PSM) list with iTRAQ labels to report the MC and IC iTRAQ labels average label intensities for each identified protein. *SignifiQuant* then uses the uTRAQ generated data to estimate which proteins are differentially expressed between two treatments, called *phenotypes*, with the least significant comparison being used to determine the proteins significance (Longworth, 2013). Here, the following settings were used: false discovery rate (FDR) = 1%; required unique peptides = 2, t-test threshold = 0.05, multiple test correction = off). The identity of the differentially expressed proteins were made by matching their accession numbers to information in the UniProt database (www.uniprot.org).

5.3.3 Identifying Unique Proteins between Treatments: Venn Diagrams

VENN diagrams were used to explore which differentially expressed proteins (DEPs) were shared among treatments and which ones were unique to specific treatments. The main goal was to specifically identify shared and exclusive proteins among and between specific treatments. First, all the combinations related to control conditions were analysed to exclude the DEPs occurring in *S. subspicatus* and not related to ASTM or DW addition; then the total overlapping DEPs were removed in the further analysis of remaining combinations, again to exclude shared DEPs but more importantly to highlight proteins unique to a given combination therefore elucidating the infochemicals response for colony formation and flocculation and distinguish from the effects caused by the infochemicals carrier (ASTM). This assessment was performed using the online tool *BioVenn*, which employs area-proportional diagrams (Hulsen et al., 2008).

5.3.4 Functional Classification and Hierarchical Clustering

Unique DEPs were then functionally classified using the KAAS - KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas), with the following settings: Search program: *BLAST*; Query sequences (in multi-FASTA): *Text data* (downloaded from UniProt); GENES data set: *manual selection → organisms list → selected organisms: Green algae, Amborella family: Chlamydomonas reinhardtii; Ostreococcus lucimarinus; Ostreococcus tauri and Micromonas commoda"*; Assignment methods: *BH (bi-directional best hit)*. KAAS results contained KO (KEGG Orthology) assignments and automatically generated KEGG pathways. KEGG identifiers were used to derive BRITE functional hierarchies (http://www.genome.jp/kegg/kegg3b.html) and reported in the supplementary material,

section V. Hierarchical clustering of the unique DEPs was based on the fold change expression values and implemented in R using the package *pheatmap.*

5.4 RESULTS

For iTRAQ#1 experiment (+2h) a total of 46,720 MS/MS scans were registered, along with 465 protein groups identified, while 47,346 MS/MS and 452 protein groups were observed for iTRAQ#2 experiment (+20h). As *S. subspicatus* is not a model organism and its genome is not sequenced yet, it was necessary to match spectra to the proteomes of a series of closely-related organisms to successfully generate hypothesis related to infochemicals response. The most reference proteomes that were identified were *Tetradesmus obliquus* (previously reported as *Scenedesmus obliquus*), several other *Scenedesmus* spp, i.e. *S. armatus*, *S. acutus*, *S. quadricauda* and *S. bijugus*, *Chlamydomonas reinhardtii*, *Chlorella variabilis, Volvox carteri f. nagariensis, Dunaliella salina, Dunaliella tertiolecta, Coccomyxa subellipsoidea and Bathycoccus prasinos*. To less extent, reference proteomes were identified in *Ostreococcus luciminarus, Ectocarpus silicosus, Cyanophora paradoxa, Micromonas pusilla* and *Microcystis aeruginosa*.

5.4.1 PCA

Fig. 5-3 shows the PCA clustering of iTRAQ #1 and #2 datasets (+2h and +20h exposure, respectively), indicating how in both cases different treatments were clearly separated. This suggests that protein abundance changed upon exposure to infochemicals and with a good grouping in the biological replicates, indicating that the biological replicates are similar enough to allow meaningful insights from the comparison of phenotypes between groups.

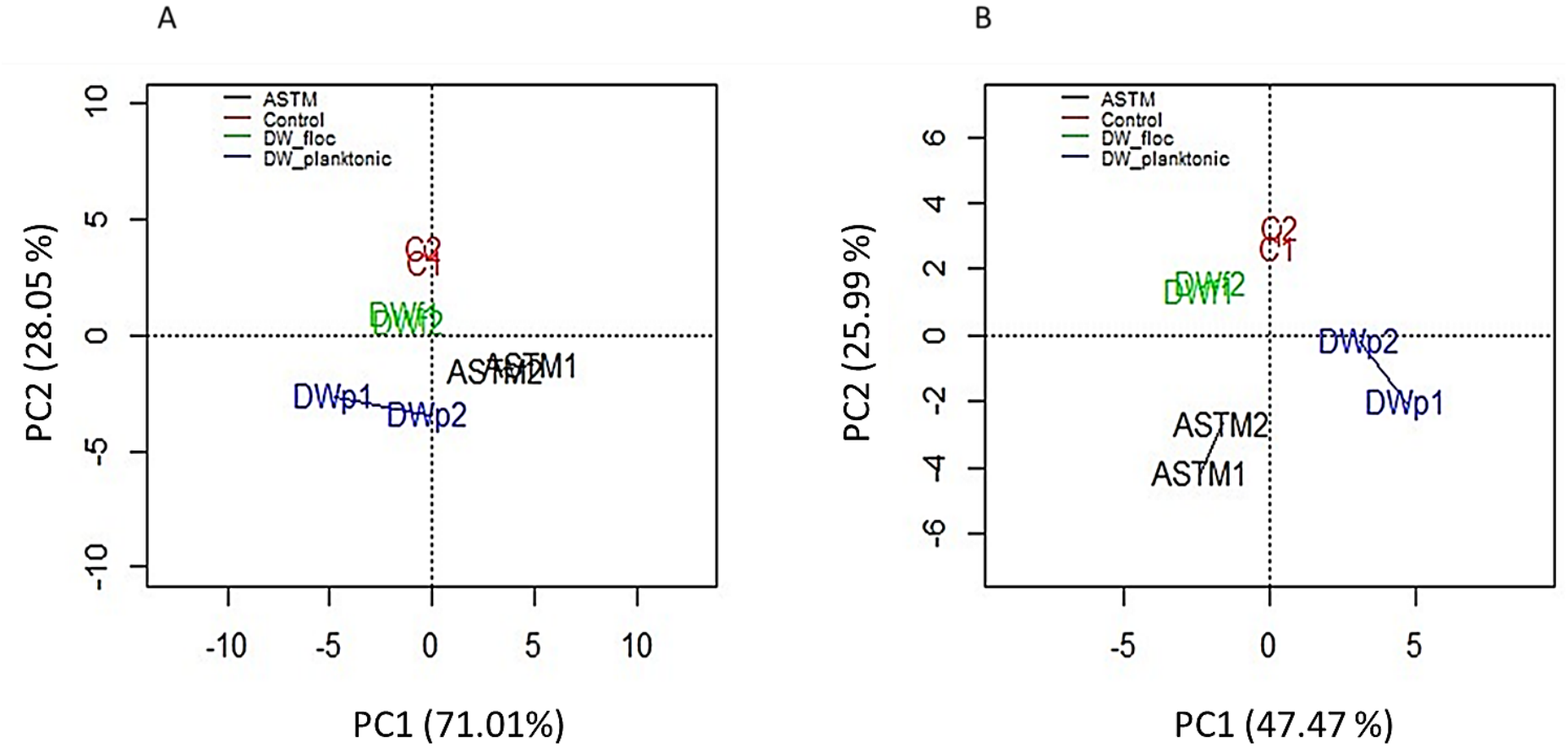


Fig. 5-3 PCA plots of the 8 samples, clustered by biological replicates. Clusters show control conditions (red), ASTM addition conditions (black), addition of DW-planktonic fraction (blue) and addition of DW-floc fraction (green). Panel A: iTRAQ#1 (+2h); Panel B: iTRAQ#2 (+20h).

Permutation based analysis of variance confirmed treatments were significantly different from Control (number of permutation= 999, iTRAQ#1 -p_{val} =0.005, iTRAQ#2 -p_{val} =0.007). The first principal component (dimension 1) accounts for as much variation in the dataset as possible (iTRAQ#1 PC1: 71%, iTRAQ#2 PC1: 47.5%); therefore top 1% contributors to PCA-dimension 1 are reported in Tables 5-2 - 5-5, with the identification of the biological process these are involved in to provide a better description of how the biological treatments are differentiated.

Table 5-2 - *Top 1% PCA contributors to dimension 1 - +2h exposure*

Entry	*Protein names*	*Organism*	*Gene ontology (biological process)*
E1ZJQ8	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial (EC 1.6.5.3) (EC 1.6.99.3) (Fragment)	Chlorella variabilis (Green alga)	Electron transport, respiratory chain
D8U1R3	Uncharacterized protein	Volvox carteri f. nagariensis	protein metabolic process [GO:0019538]
E1ZFQ1	Uncharacterized protein	Chlorella variabilis (Green alga)	metabolic process [GO:0008152]
I0YV40	Cofactor-independent phosphoglycerate mutase	Coccomyxa subellipsoidea (strain C-169) (Green microalga)	glucose catabolic process [GO:0006007]
I0YL77	ADP-ribosylation factor 1	Coccomyxa subellipsoidea (strain C-169) (Green microalga)	small GTPase mediated signal transduction [GO:0007264]
D8TIF4	Uncharacterized protein	Volvox carteri f. nagariensis	
E1ZQ02	Uncharacterized protein	Chlorella variabilis (Green alga)	proteolysis [GO:0006508]

Table 5-3 - *Top 1% PCA contributors to dimension 2 - +2h exposure*

Entry	*Protein names*	*Organism*	*Gene ontology (biological process)*
A8ISB0	Cysteine synthase (EC 2.5.1.47)	Chlamydomonas reinhardtii (Chlamydomonas smithii)	cysteine biosynthetic process from serine [GO:0006535]
D8U1R3	Uncharacterized protein	Volvox carteri f. nagariensis	protein metabolic process [GO:0019538]
A4S824	Ferredoxin-thioredoxin reductase, catalytic chain (FTR-C) (EC 1.8.7.2) (Ferredoxin-thioredoxin reductase subunit B)	Ostreococcus lucimarinus (strain CCE9901)	
C1N9S5	Heat shock protein 70 with TPR repeat	Micromonas pusilla (strain CCMP1545) (Picoplanktonic green alga)	
E1Z7R4	Heat shock protein 70	Chlorella variabilis (Green alga)	
A0A0C4K0H7	SBP protein (EC 3.1.3.37)	Dunaliella tertiolecta (Green alga)	carbohydrate metabolic process [GO:0005975]
Q9FNS5	NADP-Malate dehydrogenase (EC 1.1.1.82)	Chlamydomonas reinhardtii (Chlamydomonas smithii)	carbohydrate metabolic process [GO:0005975]; malate metabolic process [GO:0006108]; NADH metabolic process [GO:0006734]; oxaloacetate metabolic process [GO:0006107]; response to redox state [GO:0051775]; tricarboxylic acid cycle [GO:0006099]

Table 5-4 - *Top 1% PCA contributors to dimension 1 - +20h exposure*

Entry	*Protein names*	*Organism*	*Gene ontology (biological process)*
D8U1I3	Adenylosuccinate synthetase, chloroplastic (AMPSase) (AdSS) (EC 6.3.4.4) (IMP--aspartate ligase)	Volvox carteri f. nagariensis	'de novo' AMP biosynthetic process [GO:0044208]
D8U4Q1	Uncharacterized protein	Volvox carteri f. nagariensis	metabolic process [GO:0008152]
E1ZD58	Cysteine synthase (EC 2.5.1.47) (Fragment)	Chlorella variabilis (Green alga)	cysteine biosynthetic process from serine [GO:0006535]
A8IW00	Glutamine synthetase (EC 6.3.1.2)	Chlamydomonas reinhardtii (Chlamydomonas smithii)	glutamine biosynthetic process [GO:0006542]
D8TKE8	Mg-protoporphyrin IX chelatase (EC 6.6.1.1)	Volvox carteri f. nagariensis	chlorophyll biosynthetic process [GO:0015995]; photosynthesis [GO:0015979]
E1Z349	Malate dehydrogenase (EC 1.1.1.37)	Chlorella variabilis (Green alga)	carbohydrate metabolic process [GO:0005975]; malate metabolic process [GO:0006108]; tricarboxylic acid cycle [GO:0006099]
K8EQC7	Uncharacterized protein	Bathycoccus prasinos	

Table 5-5 - *Top 1% PCA contributors to dimension 2 - +20h exposure*

Entry	*Protein names*	*Organism*	*Gene ontology (biological process)*
A8IX80	Acetohydroxyacid dehydratase	Chlamydomonas reinhardtii (Chlamydomonas smithii)	branched-chain amino acid biosynthetic process [GO:0009082]; response to salt stress [GO:0009651]; root development [GO:0048364]
D8TZU3	Uncharacterized protein	Volvox carteri f. nagariensis	
I0YKI7	Heat shock protein 70	Coccomyxa subellipsoidea (strain C-169) (Green microalga)	
A8J906	Predicted protein	Chlamydomonas reinhardtii (Chlamydomonas smithii)	
I0YLA9	Prohibitin	Coccomyxa subellipsoidea (strain C-169) (Green microalga)	
Q75VY8	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii (Chlamydomonas smithii)	photosynthesis, light harvesting in photosystem I [GO:0009768]; protein-chromophore linkage [GO:0018298]; response to light stimulus [GO:0009416]

5.4.2 Phenotypes Comparison and Analysis of Differentially Expressed Proteins (DEPs)

Data were run through in-house programs *uTRAQ* and *SignifiQuant*. Comparisons were made between iTRAQ treatment groups, and *SignifiQuant* gave results for proteins which were significantly different in abundance between the phenotypes. Results are reported for iTRAQ#1 and #2 (see supporting material section III).

5.4.3 Venn Diagrams

The Venn diagrams of the DEPs are presented in Fig. 5-4, Panel A/B for iTRAQ#1 and Panel C/D for iTRAQ#2. The sum of the numbers in each large circle presents the total number of DEPs among various combinations while the overlapping parts of the circles show common differentially expressed proteins between combinations (Table 5-4). Unique DEPs for each phenotype comparison fell into four main categories: Metabolism, Cellular Processes, Genetic Information Processing and Environmental Information Processing (see supporting material section IV).

Table 5-4 *Number of Unique DEPs for each phenotypes comparison and at two different times of exposure*

Time Point	*Phenotypes Comparison*	*Unique DEPs*
+2 h (iTRAQ#1)	DW floc vs. Control	18
	DW plk vs. Control	8
	ASTM vs Control	30
	DW floc vs DW plk	6
	DW floc vs ASTM	21
	DW plk vs ASTM	2
+20 h (iTRAQ#2)	DW floc vs. Control	12
	DW plk vs. Control	14
	ASTM vs Control	23
	DW floc vs DW plk	14
	DW floc vs ASTM	14
	DW plk vs ASTM	6

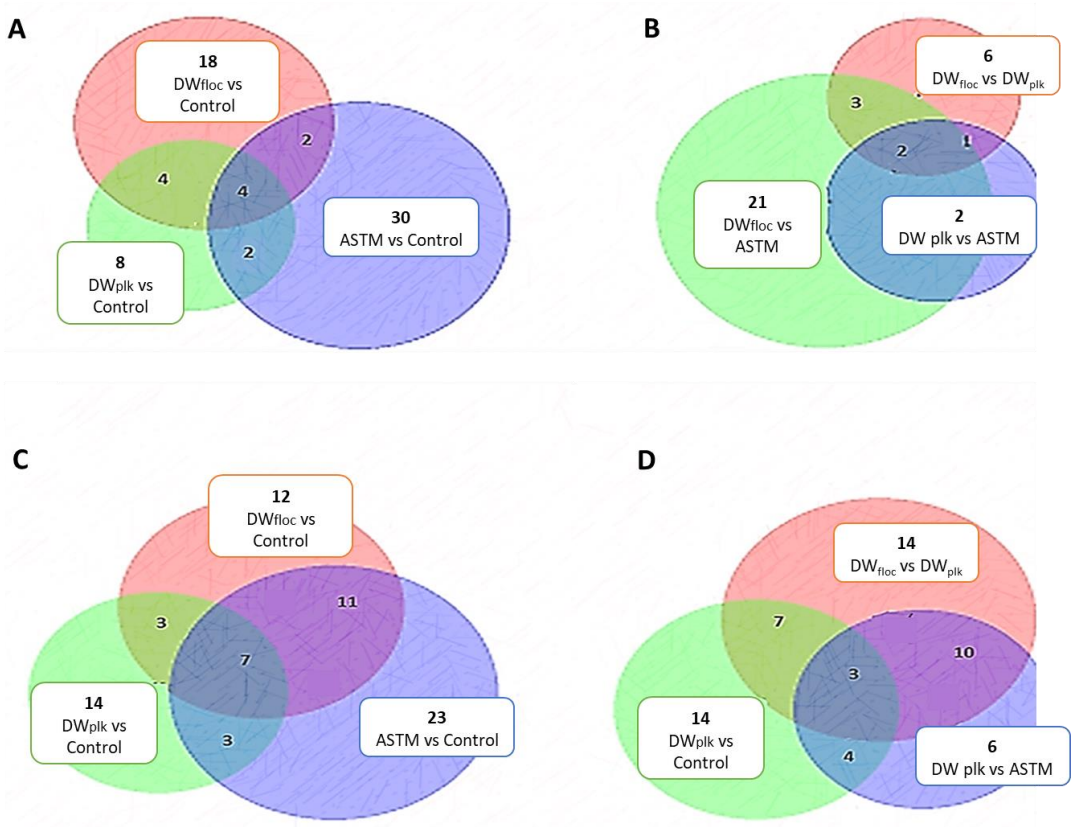


Fig. 5-4 Venn Diagrams of DEPs. Panels A/B: iTRAQ#1; Panels C/D: iTRAQ#2.

5.4.4 Hierarchical Clustering

Hierarchical clustering is a powerful tool to investigate regulatory mechanisms linked to a condition, as group proteins and samples are grouped together based on the similarity of their expression patterns and across treatments. In this study, unique DEPs were classified according to their biological functions into the following main categories: Energy, Carbohydrates and Lipids metabolism (Fig. 5-5).

At an early exposure to infochemicals (Fig. 5-5, A-C) hierarchical clustering for energy metabolism, which included photosynthesis, sulphur metabolism, carbon fixation in photosynthetic organisms and oxidative phosphorylation (see supporting material, section V), showed two main clusters: 1) DW-floc fraction against ASTM exposed cells and 2) DW (both planktonic and floc fractions) against Control. For both clusters, unique DEPs showed higher abundance. For carbohydrates metabolism, which included glyoxylate and dicarboxylate metabolism, glycolysis/gluconeogenesis, citrate cycle (TCA) and the pentose phosphate pathway (see supporting material, section V), it was shown how the proteomes of DW-planktonic fraction against either ASTM or Control were clustered together, as it was for DW-floc fraction against ASTM/Control. Also, every phenotypes comparison displayed mostly less abundance of proteins (Table 5.5).

Table 5.5 Enrichment annotations for hierarchical clustering. Alarm phase (iTRAQ#1)

Time Point	Phenotypes	*UniProt ID*	*Fold Change*	*ko list*	*Brite Hierarchy*		
2h	*DW floc vs Control*	Photosystem I P700 chlorophyll a apoprotein A1 (EC 1.97.1.12) (PSI-A) (PsaA)	2.28	K02689	Metabolism	Energy metabolism	Photosynthesis
		GTP-binding protein YPTC1	1.37	K07874	Genetic Information Processing	Folding, sorting, and degradation	Membrane trafficking
		Uncharacterized protein					
		40S ribosomal protein S5	1.31	K02989	Genetic Information Processing	Translation	Ribosome
		Elongation factor 2	1.29	K03234	Environmental Information Processing	Signal transduction	AMPK signalling pathway
		Chlorophyll a-b binding protein, chloroplastic	1.23	K08916	Metabolism	Energy metabolism	Photosynthesis
		Cysteine synthase (EC 2.5.1.47) (Fragment)	1.22	K01738	Metabolism	Energy metabolism	Sulfur metabolism
		Uncharacterized protein					
		Predicted protein (Fragment)					
		Ubiquinol: cytochrome c oxidoreductase 50 kDa core 1 subunit	-1.15	K17732	Metabolism	Enzyme families	Peptidases
		Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit (Fragment)	-1.11	K01601	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
		Uncharacterized protein					
		Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.-)	-1.22	K00627	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
		Ferredoxin-dependent glutamate synthase	-1.23	K00284	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
		Chloroplast ATP synthase gamma chain protein (Fragment)	-1.25	K02115	Metabolism	Energy metabolism	Oxidative phosphorylation
		Uncharacterized protein	-1.33	K03125	Genetic Information Processing	Transcription	Basal transcriptionfactors
		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)					
		Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	1.57	K02703	Metabolism	Energy metabolism	Photosynthesis
		Histone H2B (Fragment)	1.45	K11252	Cellular Processes	Transport and catabolism	Exosome
		Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	1.46	K02705	Metabolism	Energy metabolism	Photosynthesis
		Uncharacterized protein	1.38	K02	Metabolism	Energy metabolism	Photosynthesis

DW plk vs Control	40S ribosomal protein S6	1.32	K02692	Environmental Information Processing	Signal transduction	Apelin signalling pathway
	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	1.00	K00991	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	Uncharacterized protein	-1.09	K01031	Metabolism	Energy metabolism	Carbon fixation in photosynthetic organisms
	Uncharacterized protein	-1.13	K13100	Spliceosome	Other splicing related proteins	Spliceosome associated proteins (SAPs)
ASTM vs Control	Uncharacterized protein	1.85	K03199	Genetic Information Processing	Translation	RNAtransport
	Catalase (EC 1.11.1.6)	1.65	K03231	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	Vitamin B6 biosynthesis protein	1.53	K03781	Metabolism	Metabolism of cofactors and vitamins	Vitamin B6 metabolism
	Uncharacterized protein	1.50	K06215	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	HSP70bf (Heat shock protein 70B)	1.41	K00026	Environmental Information Processing	Signal transduction	MAPKsignalingpathway
	Peptidylprolyl isomerase (EC 5.2.1.8)	1.37	K03283	Genetic Information Processing	Folding sorting and degradation	Chaperones and folding catalysts
	Uncharacterized protein	1.37	K09568	Metabolism	Carbohydrate metabolism	Pentose phosphate pathway
	Elongation factor Tu	1.27	K01807	Cellular Processes	Transport and catabolism	Exosome
	Uncharacterized protein	1.23	K02358	Genetic Information Processing	Folding sorting and degradation	Chaperones and folding catalysts
	ATP synthase subunit beta (EC 3.6.3.14)	1.22	K03696	Genetic Information Processing	Translation	Mitochondrial biogenesis
	SBP protein (EC 3.1.3.37)	1.22	K02133	Metabolism	Energy metabolism	Carbon fixation in photosynthetic organisms
	40S ribosomal protein S12	1.22	K01100	Genetic Information Processing	Translation	Ribosome
	Molecular chaperones HSP70/HSC70, HSP70 superfamily	1.22	K02951	Metabolism	Enzyme families	Protein phosphatase and associated proteins
			K03283			

	14-3-3 protein	1.20	K06630	Genetic Information Processing	Replication and repair	DNArepairandrecombinationproteins
	Elongation factor Tu, chloroplastic (EF-Tu) Elongation factor Tu (Fragment) Phycocyanin beta subunit Flavoprotein	1.20	K02358	CellularProcesses	Transport and catabolism	Exosome
	Heat shock protein 70C	1.15	K04043	Genetic Information Processing	Folding sorting and degradation	RNA degradation
	Acetohydroxyacid dehydratase	-1.16	K01687	Metabolism	Aminoacid metabolism	Valine, leucine and isoleucine biosynthesis
	Fructose-bisphosphate aldolase 1, chloroplastic (EC 4.1.2.13)	-1.23	K01623	Metabolism	Carbohydrate metabolism	GlycolysisGluconeogenesis
	Glutamine synthetase (EC 6.3.1.2)	-1.27	K01915	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	Glucose-6-phosphate isomerase (EC 5.3.1.9)	-1.34	K01810	Metabolism	Carbohydrate metabolism	GlycolysisGluconeogenesis
	ATP synthase subunit beta (EC 3.6.3.14) (Fragment) Glyoxalase I	-1.35	K02112	Metabolism	Energymetabolism	Oxidative phosphorylation
	Malate dehydrogenase (EC 1.1.1.37)	-1.51	K00026	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	Enolase	-1.58	K01689	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Predicted protein	-1.65	K06972	Metabolism	Enzyme families	Peptidases
	Uncharacterized protein	-1.79	K04077	Genetic Information Processing	Folding sorting and degradation	RNAdegradation
	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase A) (GAPDHA) (Fragment)	-2.00	K05298	Metabolism	Energy metabolism	Carbon fixation in photosynthetic organisms
DW floc vs ASTM	ATP synthase subunit beta (EC 3.6.3.14)	1.22	K02133	Metabolism	Energy metabolism	Oxidativephosphorylation
	Elongation factor 2	1.36	K03234	Environmental Information Processing	Signal transduction	AMPKsignalingpathway
	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	1.25	K00031	Metabolism	Carbohydrate metabolism	Citratecycle(TCAcycle)
	ATP synthase subunit beta (EC 3.6.3.14)	1.22	K02133	Metabolism	Energy metabolism	Oxidative phosphorylation
	Eukaryotic translation elongation factor 1 alpha 2	1.33	K03231	Genetic Information Processing	Translation	RNA transport
	Cysteine synthase (EC 2.5.1.47)	1.16	K01	Metabolism	Energy metabolism	Sulfurmetabolism

	ATP synthase subunit alpha	1.11	738 K02 111	Metabolism	Energy metabolism	Oxidative phosphorylation
	Enolase	-1.13	K01 689	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Heat shock protein 70C	-1.19	K04 043	Genetic Information Processing	Folding sorting and degradation	RNAdegradation
	Catalase (EC 1.11.1.6)	-1.21	K03 781	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	Elongation factor Tu	-1.22	K02 358	CellularProcesses	Transport and catabolism	Exosome
	Ferredoxin-dependent glutamate synthase	-1.24	K00 284	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	Uncharacterized protein	-1.25	K00 026	Metabolism	Carbohydrate metabolism	Citratecycle(TCAcycle)
	Fructose-bisphosphate aldolase 1, chloroplastic (EC 4.1.2.13)	-1.25	K01 623	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Glutamine synthetase (EC 6.3.1.2)	-1.25	K01 915	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	Molecular chaperones HSP70/HSC70, HSP70 superfamily	-1.28	K03 283	Environmental Information Processing	Signal transduction	MAPKsignaling pathway
	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	-1.36	K02 705	Metabolism	Energymetabolism	Photosynthesis
	ATP synthase subunit beta (EC 3.6.3.14) (Fragment)	-1.42	K02 112	Metabolism	Energymetabolism	Oxidative phosphorylation
	Uncharacterized protein	-1.42	K03 125	Genetic Information Processing	Transcription	Basa ltranscription factors
	Fructose-bisphosphate aldolase (EC 4.1.2.13)	-1.49	K01 623	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.-)	-1.72	K00 627	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
DWplk vs ASTM	Cytochrome b6	2.35	K02 635	Metabolism	Energy metabolism	Photosynthesis
	Uncharacterized protein	1.43	K03 234	Environmental Information Processing	Signal transduction	MAPK signalling pathway
	Uncharacterized protein	-1.16	K01 807	Metabolism	Carbohydrate metabolism	Pentose phosphate pathway

After a longer exposure (Fig. 5-5 D-G) and for energy metabolism, unique DEPs related to DW-floc fraction against either control or ASTM or DW-planktonic fraction were more abundant, while unique DEPs linked to DW-planktonic fraction were less abundant when compared against both Control and ASTM unique DEPs. For carbohydrates metabolism, which accounted for glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, glycolysis/gluconeogenesis, citrate cycle (TCA), pentose phosphate pathway and starch and sucrose metabolism, proteins were less abundant for every phenotypes comparison. At this time of exposure, it was possible to observe the additional category of lipids metabolism, comprisive of biosynthesis of fatty acids, and which showed higher abundance of DEPs associated to planktonic cells exposed to infochemicals (Table 5.6)

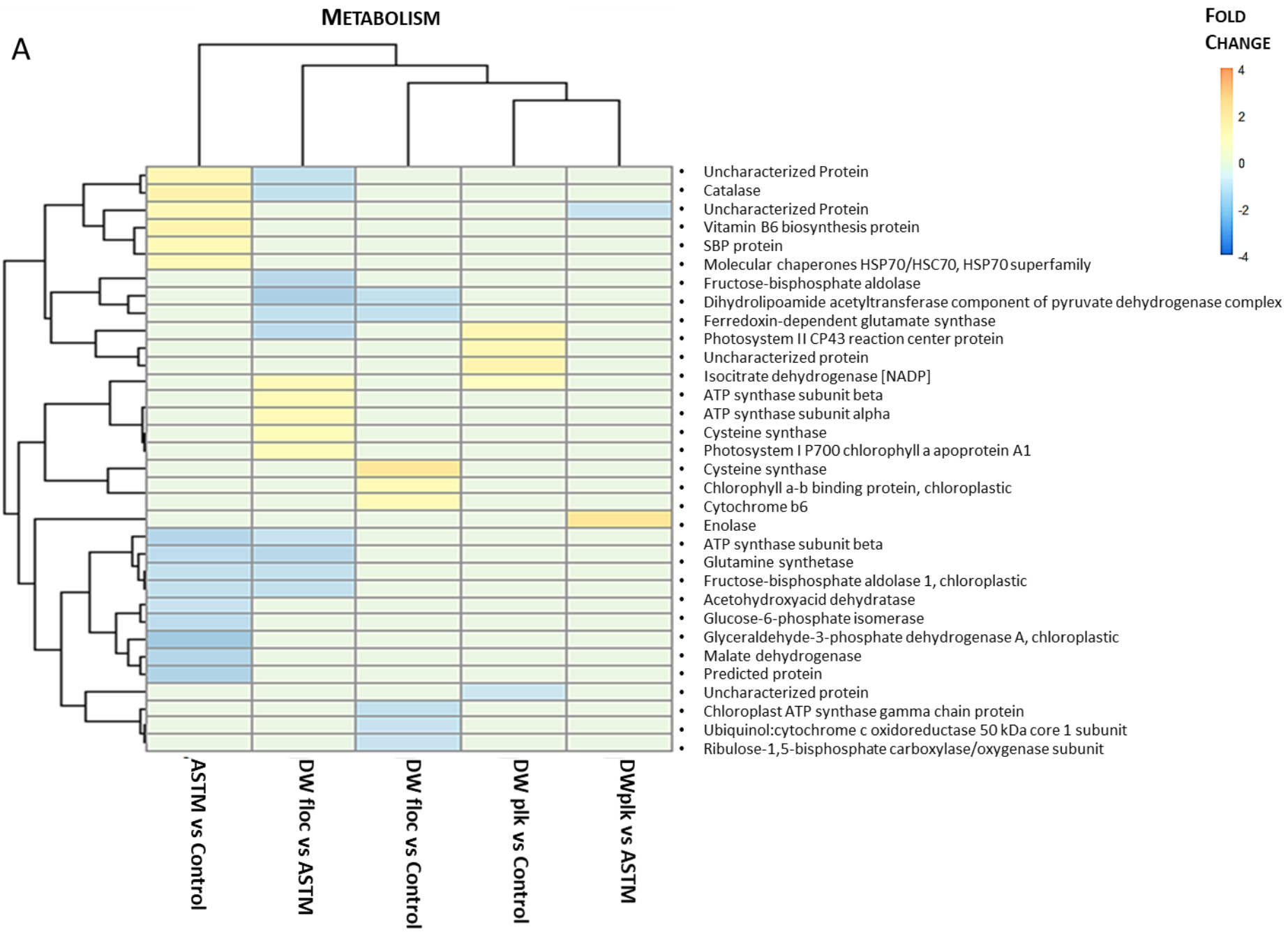
Table 5.6 Enrichment annotations for hierarchical clustering. Acclimation phase (iTRAQ#2)

Time Point	*Phenotypes*	*UniProt ID*	*Fold Change*	*ko list*	*BriteHierarchy*		
	DW floc vs Control	P37255	2.57	K02704	Metabolism	Energy metabolism	Photosynthesis
		A8HXL8	1.64	K02115	Metabolism	Energy metabolism	Oxidative phosphorylation
		I0Z5X3	1.60	K00413	Metabolism	Energy metabolism	Oxidative phosphorylation
		P10898	2.16	K02705	Metabolism	Energy metabolism	Photosynthesis
		A8JJV5	1.43	K11252	Cellular Processes	Transport and catabolism	Exosome
		A8J1G8	1.38	K02991	Environmental Information Processing	Signal transduction	Apelin signalling pathway
		C1MYV3	1.24	K10355	Cellular Processes	Cell mobility	Cytoskeleton proteins
		D8UHN1	-1.22	K01586	Metabolism	Aminoacid metabolism	Lysine biosynthesis
		A8JDW2					
		A8JCY4	-1.29	K01623	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
		I3UMQ3	-1.37	K01601	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
		I3UMR2	-1.37	K01601	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylatemetabolism
	DW plk vs Control	Q8LRU1	2.27	K00522	Cellular Processes	Cell growth and death	Ferroptosis
		Q1KVS9	2.14	K02358	Cellular Processes	Transport and catabolism	Exosome
		D8UI03	1.83	K03283	Environmental Information Processing	Signal transduction	MAPK signalling pathway
		E1ZQL8	1.75	K01845	Metabolism	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
		E1Z5I7	1.34	K08770	Genetic Information Processing	Folding sorting and degradation	Ubiquitin system
		E1ZMW8	1.28	K19269	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
		I0YZE5	1.22	K02183	Environmental Information Processing	Signal transduction	MAPK signalling pathway
		A8J6C7	1.20	K03798	Metabolism	Enzyme families	Peptidases
		E1Z6L2	-1.14	K0110	Metabolism	Energy metabolism	Carbon fixation in photosynthetic

			0			organisms
	Q84X75	-1.44	K00059	Metabolism	Lipid metabolism	Fatty acids biosynthesis
	D8UBQ8	-1.53	K00975	Metabolism	Carbohydrate metabolism	Starch and sucrose metabolism
	I0YP36	-2.13	K00031	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	C1MXS6	-2.27	K09539	Genetic Information Processing	Translation	Mitochondrial biogenesis
	B7TJI2					
ASTM vs Control	Q8HDG4	1.57	K01807	Metabolism	Carbohydrate metabolism	Pentose phosphate pathway
	D8TV46	1.37	K00026	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	A4S0V1	1.37	K04043	Genetic Information Processing	Folding sorting and degradation	RNA degradation
	A8IZU0	1.31	K09568	Genetic Information Processing	Folding sorting and degradation	Chaperones and folding catalysts
	A0A0S1LH61	1.29	K00026	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	A4RTP0	1.29	K01623	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Q42690	1.26	K01915	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	A8IW00	1.25	K02112	Metabolism	Energy metabolism	Oxidative phosphorylation
	P06541	1.23	K01689	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Q84RL9	1.21	K04564	Environmental Information Processing	Signal transduction	SOD2; superoxidedismutase, Fe-Mnfamily
	A0A1B0VE51	1.19	K03283	Metabolism	Enzyme families	Protein phosphatase and associated proteins
	D7FK90	1.18	K03526	Metabolism	Metabolism of terpenoids and polyketides	Terpenoid backbone biosynthesis
	D8U477	1.15	K01703	Metabolism	Carbohydrate metabolism	C5-Branched dibasic acid metabolism
	D8U5B1	-1.10	K03234	Environmental Information Processing	Signal transduction	AMPK signalling pathway
	I0YUW3	-1.26	K09458	Metabolism	Lipid metabolism	Fatty acids biosynthesis
	K8F4N5	-1.27	K00284	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	I0Z401	-1.28	K03231	Genetic Information Processing	Translation	RNA transport
	E1ZBK2	-1.29	K1749	Metabolism	Carbohydrate metabolism	Fructose and mannose metabolism

			7			
	A4RQS5	-1.33	K01810	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	P48101	-1.35	K03231	Genetic Information Processing	Translation	RNA transport
	D8TK12	-1.48	K01100	Metabolism	Energy metabolism	Carbon fixation in photosynthetic organisms
	D8TNN3 D8TJY9					
DW floc vs DW plk	I0YP36	2.05	K00031	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	D7FUD3	1.82	K20196	Cellular Processes	Cell motility	Cytoskeleton proteins
	A8J1G8	1.42	K02991	Environmental Information Processing	Signal transduction	Apelins ignaling pathway
	A8JJV5	1.42	K11252	Cellular Processes	Transport and catabolism	Exosome
	Q8HDD7	1.37	K02690	Metabolism	Energy metabolism	Photosynthesis
	Q1HVA2	1.33	K05298	Metabolism	Energymetabolism	Carbon fixation in photosynthetic organisms
	E1ZD58	1.28	K01738	Metabolism	Energy metabolism	Sulfur metabolism
	K8ENF9	1.28	K03283	Environmental Information Processing	Signal transduction	MAPK signalling pathway
	Q1KVW6	1.27	K02706	Metabolism	Energy metabolism	Photosynthesis
	P26526	1.18	K02111	Metabolism	Energy metabolism	Oxidative phosphorylation
	D8TYV7	-1.48	K00927	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	Q9FE86	-1.48	K03386	CellularProcesses	Transport and catabolism	Exosome
	I3UMR2	-2.31	K01601	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
	I3UMQ3	-2.50	K01601	Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
DW floc vs ASTM	A8IQU3	1.50	K02133	Genetic Information Processing	Translation	Mitochondrial biogenesis
	A8HXL8	1.44	K02115	Metabolism	Energymetabolism	Oxidative phosphorylation
	A0A097PB89	1.38	K02111	Metabolism	Energymetabolism	Oxidative phosphorylation
	A8HY43	1.27	K03231	Genetic Information Processing	Translation	RNA transport

	D8TNN3	1.36	K04043	Genetic Information Processing	Folding sorting and degradation	RNA degradation
	D8UBP2	-1.22	K01689	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	A8IZU0	-1.24	K09568	Genetic Information Processing	Folding sorting and degradation	Chaperones and foldingcatalysts
	Q84RL9	-1.25	K03283	Environmental Information Processing	Signal transduction	MAPK signalling pathway
	A8J1M9	-1.25	K00026	Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)
	A0A0S1LH61	-1.28	K01623	Metabolism	Carbohydrate metabolism	Glycolysis, Gluconeogenesis
	D7FK90					
	A8JEU4					
	A4S0V1					
	A8JCY4					
DW plk vs ASTM	D8TTX1					
	D8UI03	1.88	K03283	Metabolism	Enzyme families	Protein phosphatase and associated proteins
	D8UFR3	1.80	K02951	Genetic Information Processing	Translation	Ribosome
	K8F4N5	1.22	K09458	Metabolism	Lipid metabolism	Fatty acids biosynthesis
	Q1KVY1	-1.21	K02109	Metabolism	Energy metabolism	Oxidative phosphorylation
	A0A1B0VE51	-1.21	K04564	Environmental Information Processing	Signal transduction	SOD2; superoxide dismutase, Fe-Mn family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Catalase
Uncharacterized Protein
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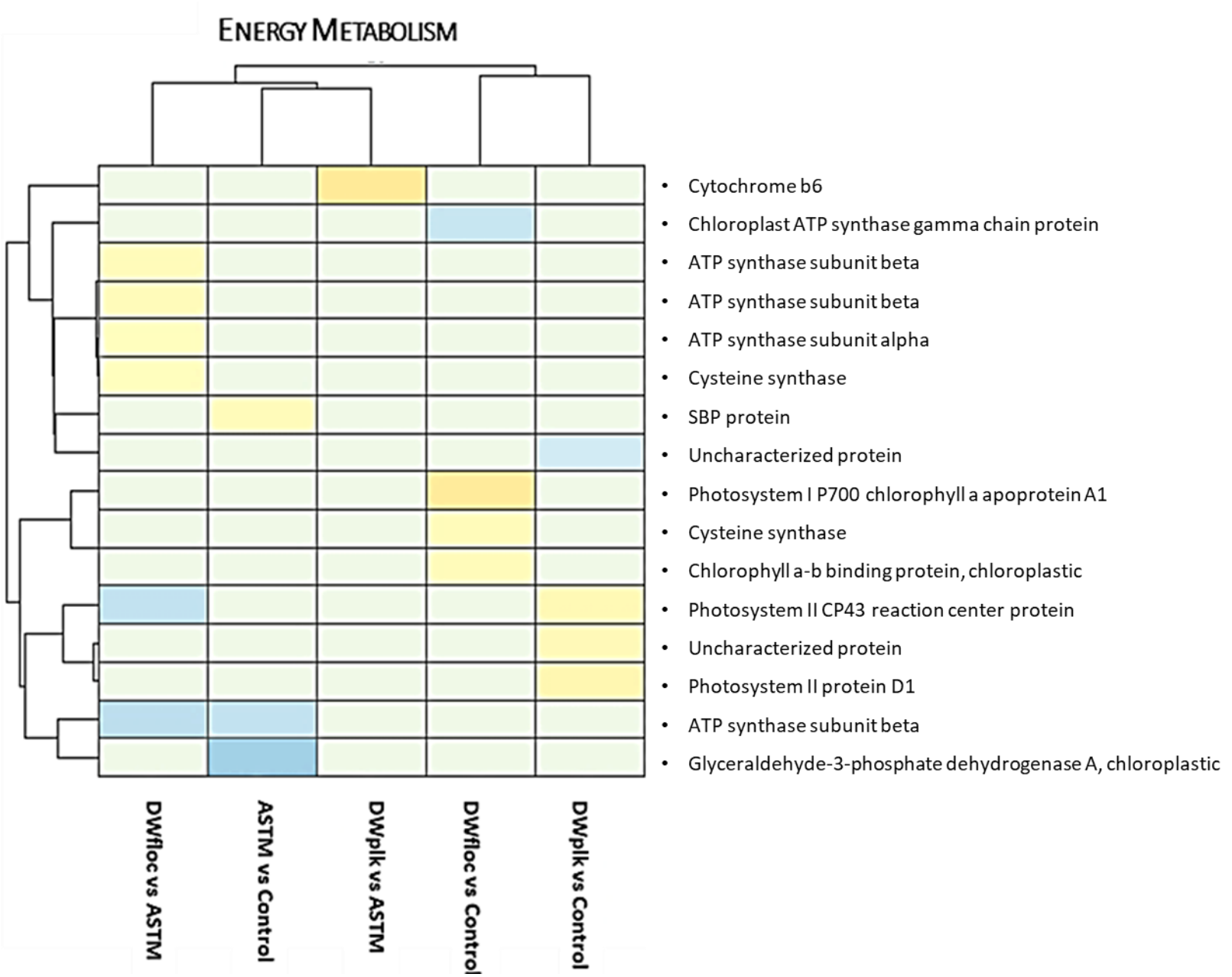
SBP protein
Molecular chaperones HSP70/HSC70, HSP70 superfamily
Fructose-bisphosphate aldolase
Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
Ferredoxin-dependent glutamate synthase
Photosystem II CP43 reaction center protein
Uncharacterized protein
Isocitrate dehydrogenase [NADP]
ATP synthase subunit beta
ATP synthase subunit alpha
Cysteine synthase
Photosystem I P700 chlorophyll a apoprotein A1
Cysteine synthase
Chlorophyll a-b binding protein, chloroplastic
Cytochrome b6
Enolase
ATP synthase subunit beta
Glutamine synthetase
Fructose-bisphosphate aldolase 1, chloroplastic
Acetohydroxyacid dehydratase
Glucose-6-phosphate isomerase
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic
Malate dehydrogenase
Predicted protein
Uncharacterized protein
Chloroplast ATP synthase gamma chain protein
Ubiquinol:cytochrome c oxidoreductase 50 kDa core 1 subunit
Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit
ASTM vs Control
DW floc vs ASTM
DW floc vs Control
DW plk vs Control
DWplk vs ASTM

B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Cysteine synthase
SBP protein
Uncharacterized protein
Photosystem I P700 chlorophyll a apoprotein A1
Cysteine synthase
Chlorophyll a-b binding protein, chloroplastic
Photosystem II CP43 reaction center protein
Uncharacterized protein
Photosystem II protein D1
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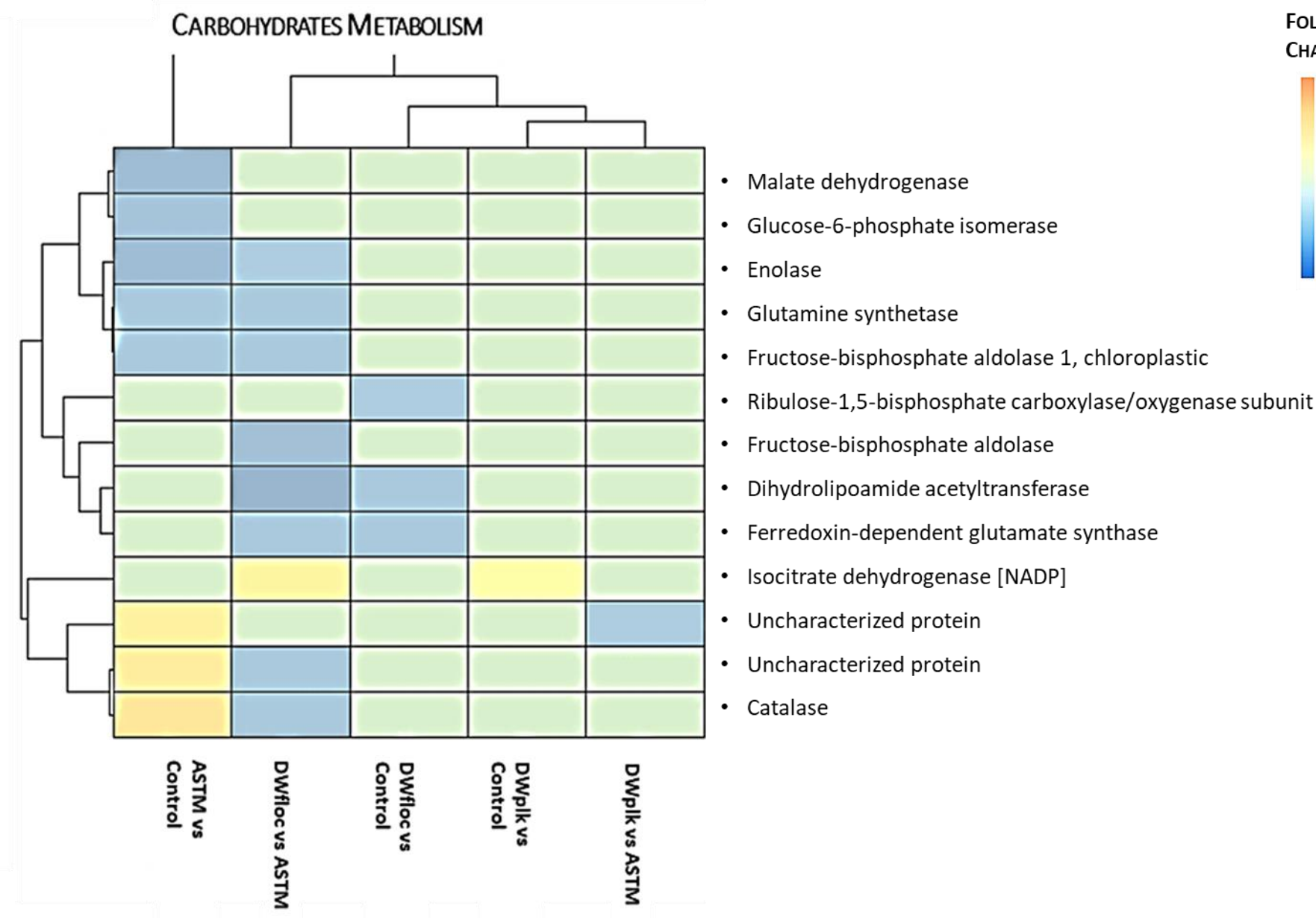
DWfloc vs ASTM
ASTM vs Control
DWplk vs ASTM
DWfloc vs Control
DWplk vs Control

C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-2
-4
ASTM vs Control
DWfloc vs ASTM
DWfloc vs Control
DWplk vs Control
DWplk vs ASTM
• Malate dehydrogenase
• Glucose-6-phosphate isomerase
• Enolase
• Glutamine synthetase
• Fructose-bisphosphate aldolase 1, chloroplastic
• Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit
• Fructose-bisphosphate aldolase
• Dihydrolipoamide acetyltransferase
• Ferredoxin-dependent glutamate synthase
• Isocitrate dehydrogenase [NADP]
• Uncharacterized protein
• Uncharacterized protein
• Catalase

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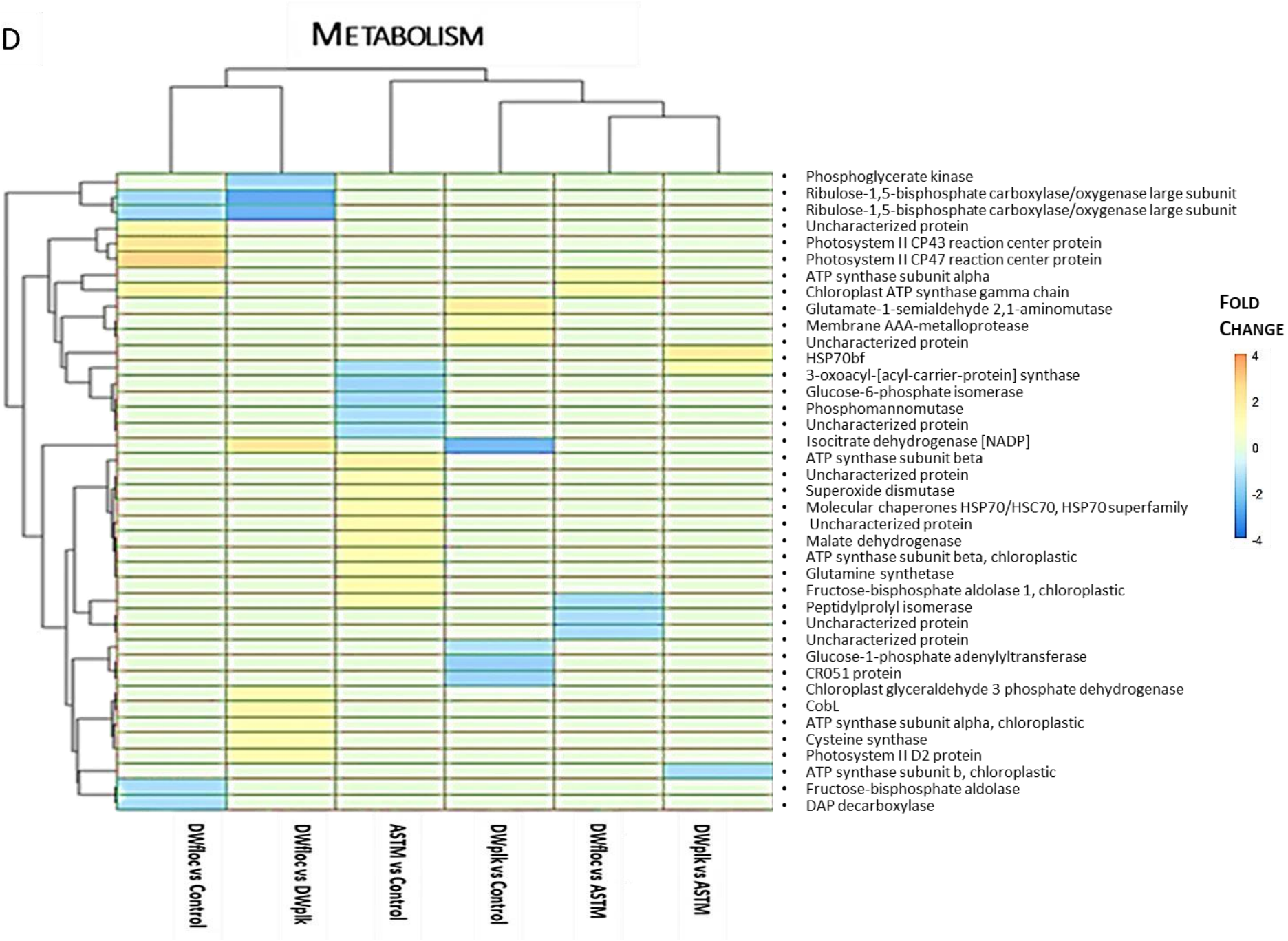
METABOLISM
DWfloc vs Control
DWfloc vs DWplk
ASTM vs Control
DWplk vs Control
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DWplk vs ASTM
Phosphoglycerate kinase
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Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
Uncharacterized protein
Photosystem II CP43 reaction center protein
Photosystem II CP47 reaction center protein
ATP synthase subunit alpha
Chloroplast ATP synthase gamma chain
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Membrane AAA-metalloprotease
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Glucose-6-phosphate isomerase
Phosphomannomutase
Uncharacterized protein
Isocitrate dehydrogenase [NADP]
ATP synthase subunit beta
Uncharacterized protein
Superoxide dismutase
Molecular chaperones HSP70/HSC70, HSP70 superfamily
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Malate dehydrogenase
ATP synthase subunit beta, chloroplastic
Glutamine synthetase
Fructose-bisphosphate aldolase 1, chloroplastic
Peptidylprolyl isomerase
Uncharacterized protein
Uncharacterized protein
Glucose-1-phosphate adenylyltransferase
CR051 protein
Chloroplast glyceraldehyde 3 phosphate dehydrogenase
CobL
ATP synthase subunit alpha, chloroplastic
Cysteine synthase
Photosystem II D2 protein
ATP synthase subunit b, chloroplastic
Fructose-bisphosphate aldolase
DAP decarboxylase
FOLD CHANGE
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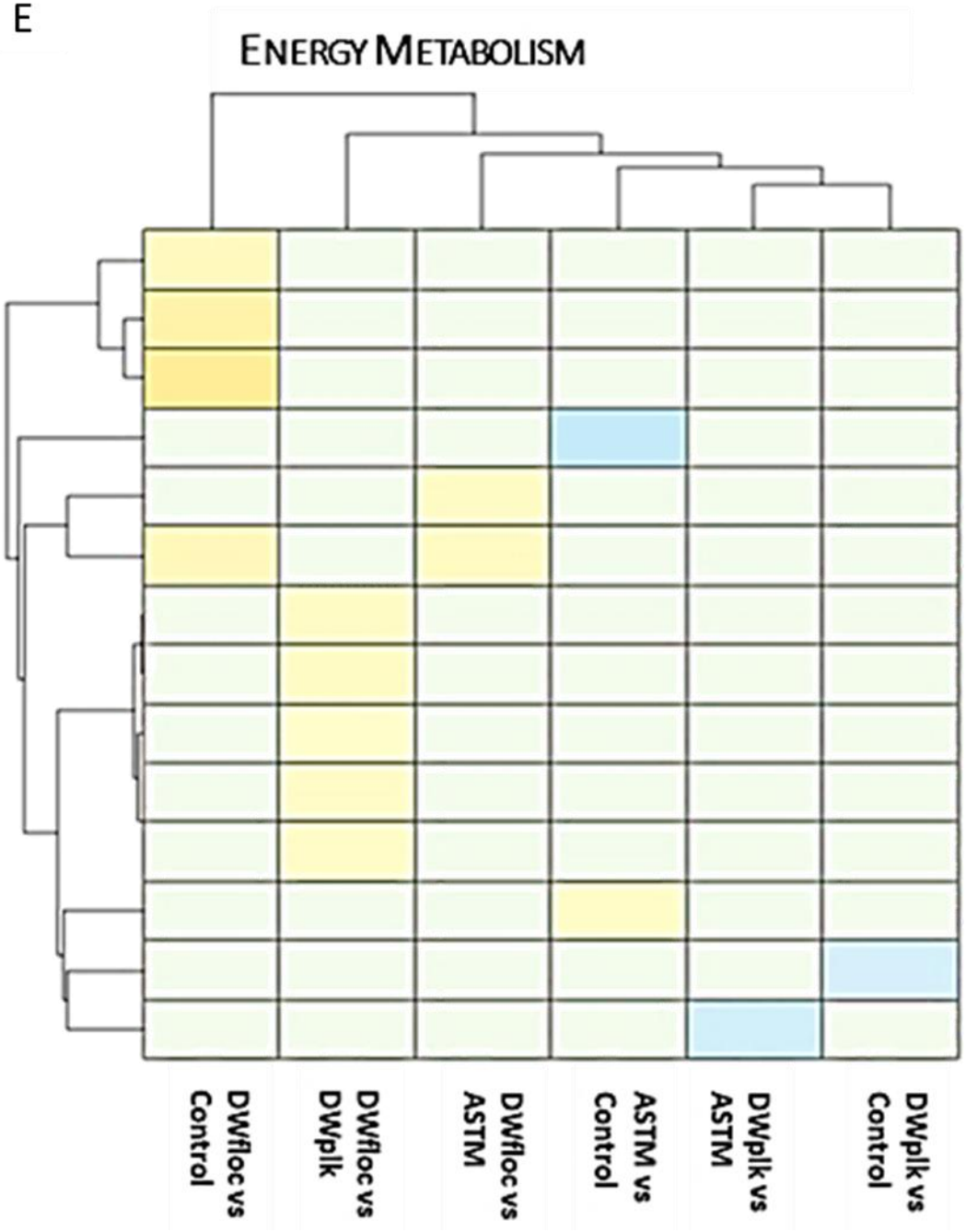
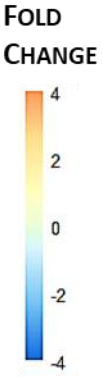
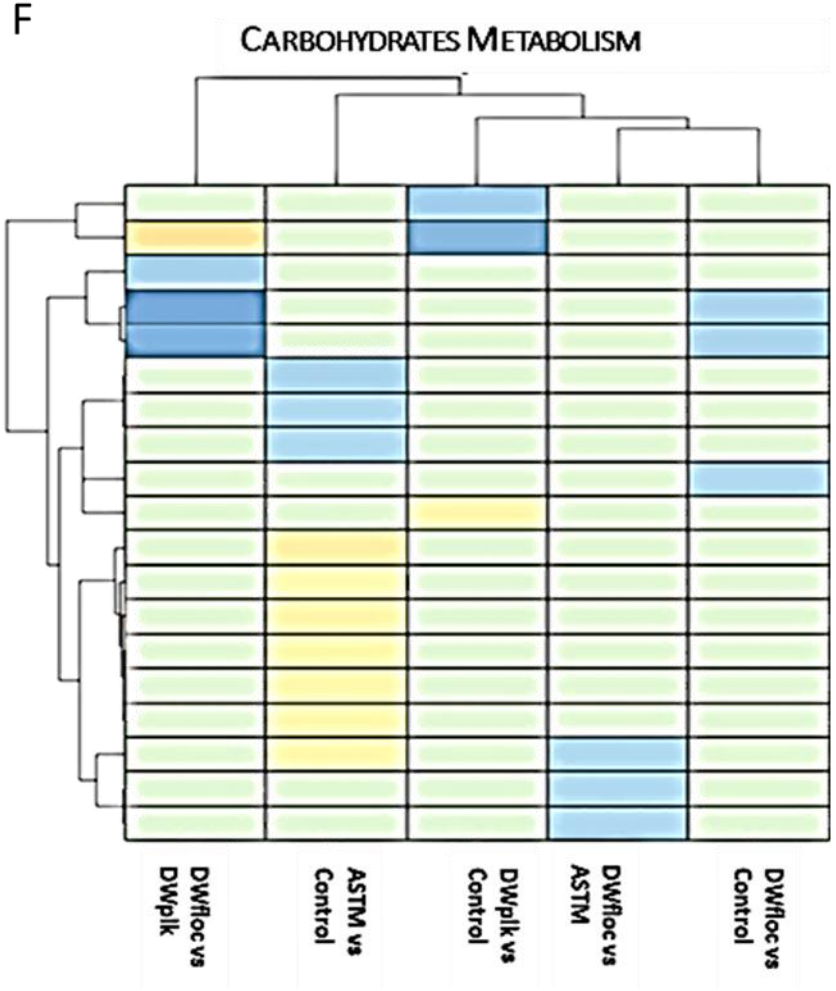
E



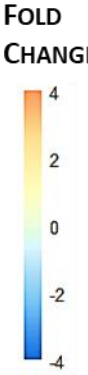
- Uncharacterized protein
- Photosystem II CP43 reaction center protein
- Photosystem II CP47 reaction center protein
- Glucose-6-phosphate isomerase
- ATP synthase subunit alpha
- Chloroplast ATP synthase gamma chain
- Chloroplast glyceraldehyde 3 phosphate dehydrogenase
- p700 chlorophyll a-apoprotein A2
- ATP synthase subunit alpha, chloroplastic
- Cysteine synthase
- Photosystem II D2 protein
- Glutamine synthetase
- Uncharacterized protein
- ATP synthase subunit b, chloroplastic



F



- Glucose-1-phosphate adenylyl transferase
- Isocitrate dehydrogenase [NADP]
- Phosphoglycerate kinase
- Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
- Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
- Phosphomannomutase
- Uncharacterized protein
- 3-oxoacyl-[acyl-carrier-protein] synthase
- Uncharacterized protein
- ATP synthase subunit beta
- Uncharacterized protein
- Uncharacterized protein
- Malate dehydrogenase
- ATP synthase subunit beta, chloroplastic
- Fructose-bisphosphate aldolase 1, chloroplastic
- Peptidylprolyl isomerase
- Thylakoid lumenal 17.4 kDa protein
- Uncharacterized protein



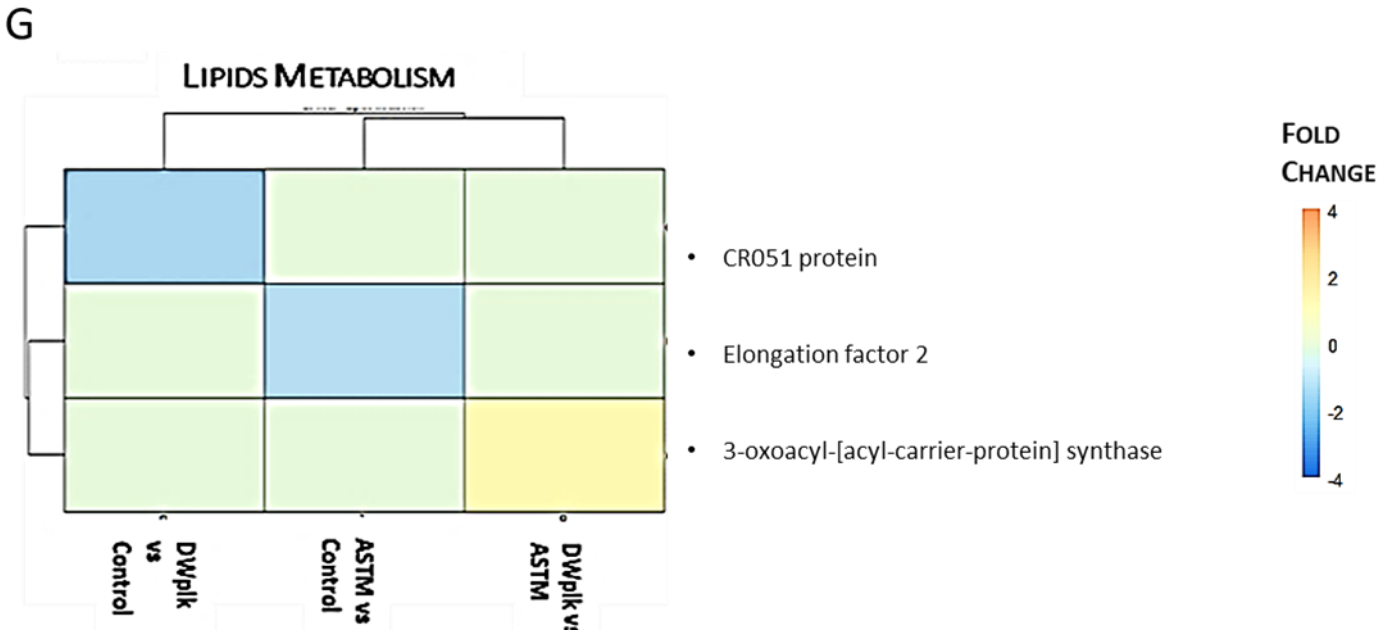


Fig. 5.5 Hierarchical clustering of unique DEPs with similar functions under infochemicals exposure.
Panel A-C: +2h and Panel D-G: +20h exposure

5.4.5 Fatty Acids

Quantification and distribution of fatty acids (FAs) in *S. subspicatus* cells exposed to *Daphnia* infochemicals, as compared to non-exposed cells, are reported in Fig. 5.6.

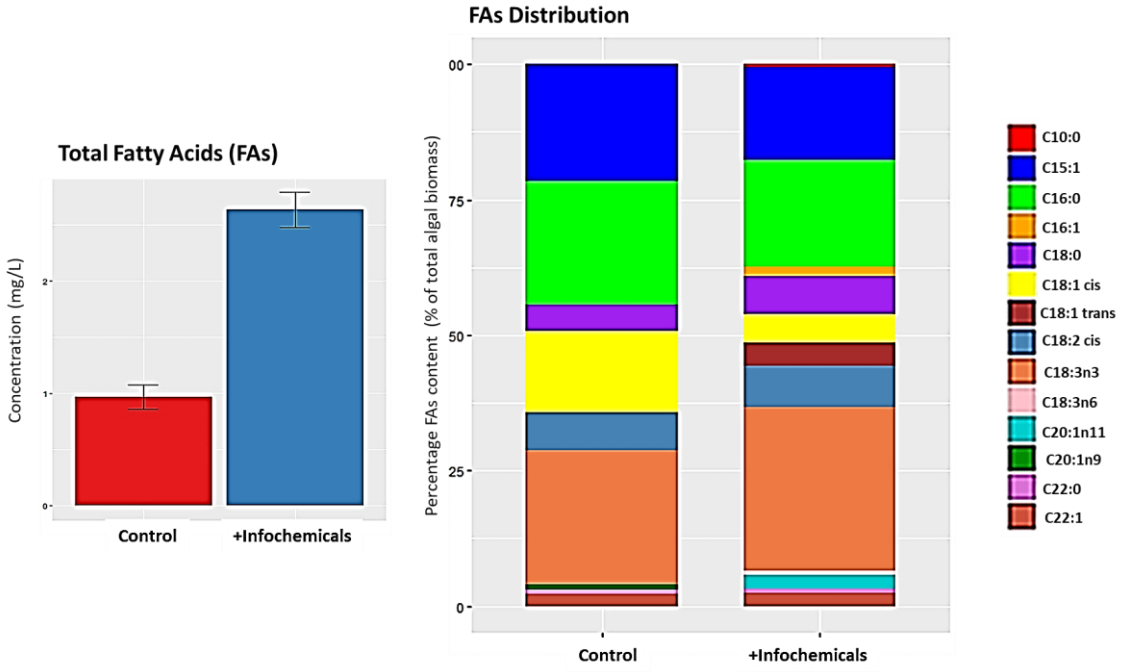


Figure 5.6 Analysis of fatty acids in *S. subspicatus* cells exposed to *Daphnia* infochemicals, as compared to non- exposed cells (Control).

The total fatty acids content almost tripled for algal cells exposed for 20h to *Daphnia* infochemicals (3mg/L). Fourteen fatty acids were identified, consisting of four unsatured (C10:0 – capric acid, C16:0 – palmitic acid, C18:0 -stearic acid, and C22 -behenic acid) and ten (mono and poly) saturated (C15:1 pentadecenoic acid- C16:1 -palmitoleic acid, C18:1 cis – oleic acid, C18:1 trans – elaidic acid, C18:2 cis – linoleic acid, C18:3n3 – alpha linoleic acid, C18:3n6 – gamma linoleic acid, C20: 1n11 – gadoleic acid, C20:1n9 – eicosenoic acid, C22:1 – erucic acid). Upon exposure to infochemicals, it was registered an increase in the relative amounts of pentadecenoic acid, oleic acid and erucic acid. Also, capric, palmitoleic and

elaidic acids were found only in infochemicals exposed cells, while gadoleic acid was only found in control algal cultures.

5.5 Discussion

Daphnia infochemicals affect the microalga *S. subspicatus* triggering defensive mechanisms which include the formation of colonies and flocculation. To date however, the cellular processes involved in this response are not well understood. Here, an in-depth, iTRAQ-based study was performend to identify proteins that are linked to grazer-infochemical induced flocculation. The experimental design allowed separation of the effects of infochemicals from the water-based carrier. The timing and mechanism of responses were further isolated by examining protein expression at two stages of algae population growth, and in floc and planktonic fractions of *S. subpsicatus*. In the following sections, the protein expression patterns induced by infochemicals at early "alarm" and late "acclimation" stages of exposure are reviewed. In each of these sections overall pattern among carbohydrates, lipid and energy metabolism and any specific protein of interest are analysed. These overviews are combined in the final section to reveal a proposed mechanism by which flocculation is occurring.

5.5.1 Alarm Phase (2h Exposure)

Protein abundance changes for both the floc and planktonic fraction of *S. subpsicatus* suggested an increased energy requirement. In particular, proteins linked to oxidative phosphorylation, providing most of the ATP needed by algae (Chen et al., 2015), were more abundant. Also, an increase in the abundance of proteins linked to photosynthesis (i.e. photosystem I P700 chlorophyll a apoprotein A1, photosystem II CP43 reaction center protein, photosystem II protein D1) was observed. In conditions

where no grazer are present, algal cells would prefer to keep their position in the upper layers of the water column, where there are more favourable conditions for growth, i.e. higher sunlight availability for photosynthesis (Lürling and van Donk, 2000). Increased photosynthesis protein abundance under grazer cue conditions in this experiement may therefore be explained by an energy-demanding diversion of algal cells efforts to compensate for a reduced access to light, as they are bigger in size and 'packed' in sinking flocs or located in inner parts of the *coenobia*.

Only in the the floc fraction at +2-hours a higher abundance of the enzyme cysteine synthase was detected; this is responsible for the formation of cysteine and is linked to the assimilation of sulphur (Vallon and Spalding, 2009, Shi et al., 2107). The significance of cysteine is linked not only to its primary role as an amino-acid due to the presence of disulphide bridges which are important contributors to the structural stability of proteins, but also to its function as a precursor to a variety of essential bio-molecules which have been linked to adaptation responses against changing environments. These include protection against oxidative stress, detoxification from xenobiotics and heavy metals as well as defence response against herbivores and pathogens, and associated to the high reactivity of the cysteine thiol group. (Romero et al., 2014, Aziz et al., 2016, Shi et al., 2017). Cysteine has also been reported to stimulate bio-flocculation of bacteria by promoting the production of extra-cellular proteins containing more disulphide bonds (Xie et al., 2013). In this study, the higher abundance of cysteine synthase in the floc fraction may therefore suggest that sulphur is required for *S. subspicatus* to flocculate as a defence response to grazers' infochemicals.

Photosynthesis is the process through which energy from light is captured to stimulate the synthesis of carbohydrates; for the floc fraction, unique DEPs linked to carbohydrates metabolism were less abundant, suggesting that algal cellular sinks might use the products of photosynthesis to boost other processes other than accumulation of carbohydrates. Several examples can be found in the literature for a reduction of carbohydates metabolism. For example, Shanmuganathan et al., in 2004 reported that *Saccharomyces cerevisiae* cells subjected to oxidative stress showed an oxidation/inactivation of glycolytic enzymes, causing a rearrangement of glucose equivalents through the pentose phosphate pathway to provide the required reducing power, in the form of NADPH (Nicotinamide Adenine Dinucleotide Phosphate), for anti-oxidant defence mechanisms. Wei et al., in 2017 also reported a reduction of carbohydrates metabolism upon palmella formation in *Dunaliella salina* following salt stress, with proteins involved in glycolysis, pentose phosphate pathway, starch mobilization and glucose metabolism. In that case, a decreased cellular carbohydrate levels corresponded to an increase in extracellular carbohydrates, indicating the activation of a mechanisms to sustain osmotic equilibrium between intra- and extra-cellular conditions.

Proteins abundance changes related to carbohydrates metabolism for the planktonic fraction showed that the enzymes isocitrate dehydrogenase [NADP] and catalase were more and less abundant, respectively, compared to control conditions. It is reported in literature that the isocitrate dehydrogenases catalyse oxidative reactions which require either NAD+ or NADP+ to produce NADH and NADPH, respectively and which are both involved in protections of cells from oxidative damage (Kil et al., 2006). During normal cell metabolism, reactive oxygen species (ROS) are inevitably produced;

these however increase under stress conditions and can work as signalling molecules to trigger cell responses (Michelet et al., 2013). The connection between ROS signalling and cellular redox have been suggested to be mediated by NADPH, among others (Mittler et al., 2009); also, ROS production could be stimulated through inhibition of the redox-sensitive enzyme catalase (Kil et al., 2006). ROS have been reported to be able to change the activity of several regulatory enzymes and in particular phosphatases like the mitogen-activated protein kinase (MAPK) phosphatases (Demidchik, 2015). In plants, ROS signalling has been linked to many other different signalling networks, including redox responses, and in some circumstances accumulation of ROS was found to either be the direct result or lead the way to signalling processes through these networks. This would be the case for the MAPK cascade (Mittler et al., 2009). Increased abundance unique DEPs for the planktonic fraction were linked to signal transduction and in particular to the MAPK class (see supporting material section V). Sensing of stressing signals and their transduction into adaptive responses is of vital importance to adapt and survive to changing conditions. In plants, MAPK pathways are connected to the regulation of growth, development and cell division, and in response to a wide range of both abiotic and biotic stimuli, including light, cold and heat, salinity, ROS or attack from pathogens (Pitzschke e al., 2009, Livanos et al., 2012). These results therefore might suggest the role of the MAPK signalling pathway in the adaptive response of *S. subpsicatus* to infochemicals triggering cell-division and therefore colonies formation.

5.5.2 Acclimation Phase (20h Exposure)

Protein abundance changes for the floc fraction at 20 hours indicated again an increased energy requirement for *S. subspicatus* in response to infochemicals;

however, the concomitant decrease of energy metabolism protein abundance for the planktonic fraction might suggest that *S. subspicatus* cells might try to minimize energy acquisition while maintaining their colonial form or alternatively divert most of their efforts to keep cells in the floc form. Also, the floc fraction kept showing a higher abundance of the enzyme cysteine synthase, hence suggesting its role in in bio-flocculation.

For carbohydrates metabolism, contrarily to what found at the alarm phase, the planktonic fraction showed a decreased abundance of the isocitrate dehydrogenase and hydrolases, while phosphatases were more abundant. As mentioned in the previous section, ROS are normally and inevitably produced because of cell metabolism, however under stress conditions their production is increased and ROS can act as signalling molecules to iniatiate cell responses (Michelet et al., 2013), modulating the activity of many regulatory enzymes including MAPK phosphatases (Demidchik, 2015). At the acclimation phase, increased abundance unique DEPs were linked to MAPK signalling cascade for both planktonic and floc fractions (see supporting material section V). In plants, MAPK pathways are involved in regulation of cell division (Livanos et al., 2012); also, it has been reported how in yeast cell-cell adhesion can be conferred by adhesins, a special class of cell wall proteins whose synthesis is controlled by various signalling cascades pathways including MAPK and in response to stress factors such as limiting nutrients conditions and/or exposure to chemical cues (Verstrepen and Klis, 2006). Braun in 2008 also reported how the genes responsible for aggregates ad biofilm formation in yeast are phenomena mediated trough MAPK pathways by extracellular cAMP (cyclic adenosine mono phosphate).

Interestingly, the first contributor to PCA-dimension 1 at the acclimation phase is the enzyme AMPSase, involved in the 'de-novo' AMP biosynthetic process (see Table 5-3). Altogether, these results could therefore suggest the role of the MAPK signalling pathway in the adaptive response of *S. subpsicatus* to infochemicals triggering and maintaining cell-division (for colony formation) and promoting flocculation (cell-cell adhesion).

Only the planktonic fraction exhibited variations in protein abundance for lipid metabolism, in the form of fatty acids biosynthesis. The proteins involved, i.e. 3-oxoacyl-[acyl-carrier-protein] synthase (inferred from *Bathycoccus prasinos*) and reductase (from *Chlamydomonas reinhardtii*) are both related to the synthesis of fatty acids (Yokoyama et al., 2001). This is a process where acyl chains are formed to be used in several end-products like cellular membranes (Chan and Vogel 2010) and contributes to the fluidity of the cell membrane, reported as an essential feature for the mobility and functionality of cellular functions, the diffusion of molecules across the membrane as well as an accurate separation of membranes during cell division (Haddaji et al., 2017). On top of their role in cellular structure, fatty acids are involved in photosynthesis (Allakhverdiev et al., 2009) and signal transduction (Graber et al., 1994). Fatty acids analysis revelaed that under the effects of predation cues *S. subspicatus* cells responded with an increase in the amount of fatty acids produced and with a redistribution of their composition, with longer acyl chains and varying degrees of saturation. The composition of fatty acids in microalgae is reported to change with changing environmental conditions to allow cells to cope with varying circumstances or trigger defence responses (Wacker et al., 2016, Darki et al., 2017), with their function being determined by length, position and saturation level of its acyl

chain (Walley et al., 2013). Li and Hu in 2005 reported how allelochemicals released from the macroalga *Phragmites communis* caused an increase in the concentration of unsaturated fatty acids lipds in the cell membrane of bloom forming species such as *C. pyrenoidosa* and *M. aeruginosa*, accompanied by a decrease in the activity of the enzyme superoxide dismutase (SOD), a metalloenzyme that converts superoxide anions to oxygen and hydrogen peroxide and playing a crucial role in defense from radicals produced during oxidative stress (Kehrer et al., 2010) as well as inhibiting membrane lipid peroxidation (Li and Hu, 2005, Wang et al., 2017). Oxidative stress conditions caused by the formation of free radicals and hydroperoxides are linked to lipid peroxidation of cellular membranes (Bhattacharya et al., 2015). This involves oxidative degradation of poly-unsaturated fatty acids and for plants it has been reported that a reduced level of saturated fatty acids and high levels of unsaturated fatty acids in membranes are caused by lipid peroxidation. Also, decreased activities of antioxidant enzymes like SOD could result in an increased level of lipid peroxidation (Bhattacharya et al., 2015). Interestingly, it was here found a decreased abundance of the enzyme Fe-SOD (Fold Change = -1.21) for the planktonic fraction of *S. subspicatus* cells exposed to *Daphnia* infochemicals. The results here presented could therefore be explained hypothesizing that under infochemicals effect, *S. subpsicatus* lipids rich in polyunsaturated fatty acids (PUFAs) might provide specific acycl groups to allow rapid adaptation of algal cell membranes (Goldber et al., 2005). The lipids most susceptible to oxidation are those having more unsaturated bonds, therefore more unsaturated fatty acids would need to be produced and integrated into cell membranes to sustain their functions (Shao et al., 2009).

5.5.3 The effect of ASTM

Surprisingly, the addition of ASTM water alone (a four salts hard artificial pond water used in standardised testing, see Chapter III) induced many protein abundance variations in *S. subpsicatus* cells. Among the unique DEPs, it was noted the presence of heat shock proteins which are linked to what is reported in literature for algae and plants responding to salt stress. In fact, Wang et al., in 2008 and Wei et al., in 2017 investigated the molecular adaptation mechanims against salinity stress of the plant *Physcomitrella patens* and the microalga *D. salina*, respectively, to report an increase in the abundance of heat shock proteins 70s (HSP70). These are molecular chaperons which play a key role in the protection of algal or plant cells through correct folding of proteins. These results would suggest that the presence of salts, despite added in low concentrations, elicits metabolic responses in *S. subpsicatus* cells, and which are different from those proteins abundance variations occurring in the presence of infochemicals. Future research should be directed towards the evaluation of the interference of salts in the infochemicals induced response in *S. subspicatus.*

5.5.4 Membrane Proteins

In the present study, the focus was on the study of soluble proteins. Although membrane proteins play pivotal roles in cellular processes, their hydrophobic properties make complete structural and functional characterization challenging. In fact, finding the appropriate detergents and buffer conditions to obtain optimal protein stability without loss of functions is often a time-consuming trial and error process (Rawlings, 2016); also, the presence of detergents is usually incompatible with the ionization methods used in mass spectrometry (Rawlings, 2016), leading to

peptides signal suppression (Bagag et al., 2013) and limiting the amount of information acquired (Schey et al., 2013).

5.5.5 Mechanisms of Infochemicals Induced Flocculation

These proteomics data indicate bio-flocculation of *S. subspicatus* in response to *Daphnia* infochemicals occur at the 2-hour, early "alarm" phase, requiring increased energy resources, and with a key role envisaged in the synthesis of cysteine, a primary amino-acid, precursors of defense biomolecules and promoter of bio-flocculation through the production of extra-cellular proteins with disulphide bonds.

Higher abundance of proteins related to photosynthesis, coupled with decreased protein abundance for carbohydrates metabolism, suggests bio-flocculation is boosted by production of different molecules other than polysaccharides and which would constitute the EPS matrix responsible for holding algal cells together. The data also suggested infochemicals induced flocculation may be sustained through MAPK signalling cascades.

As mentioned earlier in this thesis, it remains important to distinguish between flocculation and colony formation and the proteomic experimental results, contrasting floc and planktonic cell responses, support this idea that there are two separate processes. In fact, and in contrast to flocculation, colony formation required higher energy demands at the alarm phase which later decreased at the acclimation stage, therefore suggesting a trade-off between colony formation and support of floc form. Results suggested a role of fatty acids metabolism in the process of colony formation, as they contribute to the several cellular functions, including the accurate separation of membranes during cell division.

5.5.6 The Wider Perspective

Defensive responses in algae to their grazers are widely studied and represent a major interest in ecology (Lürling 2003, van Holthoon et al., 2003, Pohnert et al., 2007, O'Donnell et al., 2013, Wu et al., 2013, Zhu et al., 2015), evolution (Fischer et al., 2014) and engineering (Montemazzani et al., 2015, Alam et al., 2016, Roccuzzo et al., 2016). Where biotechnology has been the focus, authors have investigated the combined effects of nutrients or temperature manipulation with *Daphnia* infochemicals to promote colony formation in *S. obliquus* to facilitate harvesting (Zhu et al., 2015, Zhu et al., 2017). While confirming the role of these cues in the enhancement of colony formation, none of these studies performed engineering measures regarding the efficiency of the process or its feasibility, limiting their investigations in cell count variations or growth rate measurements. While being re-enforced by data throughout the present thesis, it was also shown that infochemicals induced *Scenedesmus* flocs are not formed by colonies (coenobia) but rather unicells held together, therefore highlighting 1) the need for a uniformed, standard nomenclature and 2) the distinction between the induced defences (coenobia vs. flocs) and their actual potential in biotech applications.

5.6 Conclusions and Future Perspectives

This work represents the first study combining biology, ecology and engineering approaches to unravel the molecular processes behind the response of *S. subpsicatus* to produce colonies and flocculate as an adaptive response to *Daphnia* infochemicals. These were linked to photosynthesis, carbohydrates and lipids metabolism as well as signal transduction pathways. This is particularly valuable to the algal based manufacturing industry of low-medium value products, where flocculation is a key

step to achieve economical and sustainable biomass harvesting. Infochemicals induced bio-flocculation has great potentials, as it would allow the application of a sustainable and controllable method on a large scale which also avoids metal contamination of the biomass.

Scenedesmus spp have attractive features for industrial applications; however, they do not represent model-organisms in molecular research and the use of proteomics to unravel the infochemicals response has required a combination of high quality mass spectrometry and search algorithms as well as a bit of audacity. Future research should be therefore considering matching the existing mass spectra to an up-to-date, annotated proteome database for this specific microalgal species to improve the number of proteins quantified. Moreover, future efforts should include the study of the membrane proteome of *S. subspicatus* in response to *infochemicals* to evaluate their role cellular functions like cell adhesion, molecular transport and signal transduction, therefore providing a global view of these induced responses and ultimately facilitating their incorporation into engineering practice.

REFERENCES- CHAPTER V

1. Alam MA, Vandamme D, Chun W, Zhao X, Foubert I, Wang Z, et al. Bioflocculation as an innovative harvesting strategy for microalgae. Reviews in Environmental Science and Bio-Technology. 2016;15(4):573-83.

2. Allakhverdiev SI, Los DA, Murata N. Regulatory Roles in Photosynthesis of Unsaturated Fatty Acids in Membrane Lipids. Lipids in Photosynthesis: Essential and Regulatory Functions. 2009; 30:373-88.

3. Allmer J. Algorithms for the de novo sequencing of peptides from tandem mass spectra. Expert Review of Proteomics. 2011;8(5):645-57.

4. Alonso-Gutierrez J, Kim E-M, Batth TS, Cho N, Hu Q, Chan LJG, et al. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. Metabolic Engineering. 2015; 28:123-33.

5. Armengaud J, Trapp J, Pible O, Geffard O, Chaumot A, Hartmann EM. Non-model organisms, a species endangered by proteogenomics. Journal of Proteomics. 2014; 105:5-18.

6. Aziz M, Nadipalli R, Xie X, Sun Y, Surowiec K, Zhang J-L, et al. Augmenting Sulfur Metabolism and Herbivore Defense in Arabidopsis by Bacterial Volatile Signaling. Frontiers in Plant Science. 2016;7.

7. Bagag A, Jault J-M, Sidahmed-Adrar N, Refregiers M, Giuliani A, Le Naour F. Characterization of Hydrophobic Peptides in the Presence of Detergent by Photoionization Mass Spectrometry. Plos One. 2013;8(11).

8. Baumann K, Carnicer M, Dragosits M, Graf AB, Stadlmann J, Jouhten P, et al. A

multi-level study of recombinant Pichia pastoris in different oxygen conditions. Bmc Systems Biology. 2010;4.

9. Bhattacharya P, Chakraborty N, Pal R. Bioremediation of Toxic Metals using Algae. Algal Biorefinery: an Integrated Approach: Capital Publishing Company; 2015.

10. Bogino PC, de las Mercedes Oliva M, Sorroche FG, Giordano W. The Role of Bacterial Biofilms and Surface Components in Plant-Bacterial Associations. International Journal of Molecular Sciences. 2013;14(8):15838-59.

11. Braun S. Natural Products in Mycelial Micro-organisms: Impact of morphology. In: Ikan R, editor. Selected Topics in the Chemistry of Natural Products: World Scientific Publishing Co. Pte. Ltd.; 2008.

12. Carpentier SC, Panis B, Vertommen A, Swennen R, Sergeant K, Renaut J, et al. Proteome analysis of non-model plants: A challenging but powerful approach. Mass Spectrometry Reviews. 2008;27(4):354-77.

13. Chan DI, Vogel HJ. Current understanding of fatty acid biosynthesis and the acyl carrier protein (vol 430, pg 1, 2010). Biochemical Journal. 2010; 430:559-.

14. Chen H, Hu J, Qiao Y, Chen W, Rong J, Zhang Y, et al. Ca2+-regulated cyclic electron flow supplies ATP for nitrogen starvation-induced lipid biosynthesis in green alga. Scientific Reports. 2015;5.

15. Darki BZ, Seyfabadi J, Fayazi S. Effect of nutrients on total lipid content and fatty acids profile of Scenedesmus obliquus. Brazilian Archives of Biology and Technology. 2017;60.

16. Demidchik V. Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. Environmental and Experimental Botany. 2015; 109:212-28.

17. Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N, et al. An insight into iTRAQ: where do we stand now? Analytical and bioanalytical chemistry. 2012;404(4):1011-27.

18. Fischer BB, Kwiatkowski M, Ackermann M, Krismer J, Roffler S, Suter MJF, et al. Phenotypic plasticity influences the eco-evolutionary dynamics of a predator-prey system. Ecology. 2014;95(11):3080-92.

19. Gonneaud A, Asselin C, Boudreau F, Boisvert F-M. Phenotypic Analysis of Organoids by Proteomics. Proteomics. 2017;17(20).

20. Graber R, Sumida C, Nunez EA. Fatty-Acids Analysis and Cell Signal Transduction Journal of Lipid Mediators and Cell Signalling. 1994;9(2):91-116.

21. Gulez G, Altintas A, Fazli M, Dechesne A, Workman CT, Tolker-Nielsen T, et al. Colony morphology and transcriptome profiling of Pseudomonas putida KT2440 and its mutants deficient in alginate or all EPS synthesis under controlled matric potentials. Microbiologyopen. 2014;3(4):457-69.

22. Haddaji N, Mahdhi AK, Ben Ismaiil M, Bakhrouf A. Effect of environmental stress on cell surface and membrane fatty acids of Lactobacillus plantarum. Archives of Microbiology. 2017;199(9):1243-50.

23. Harke MJ, Jankowiak JG, Morrell BK, Gobler CJ. Transcriptomic Responses in the Bloom-Forming Cyanobacterium Microcystis Induced during Exposure to Zooplankton. Applied and Environmental Microbiology. 2017;85(4):e02832-16.

24. Helliwell KE, Pandhal J, Cooper MB, Longworth J, Kudahl UJ, Russo DA, et al. Quantitative proteomics of a B12 -dependent alga grown in coculture with bacteria reveals metabolic tradeoffs required for mutualism. The New phytologist. 2017.

25. Hulsen T, de Vlieg J, Alkema W. BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. Bmc Genomics. 2008;9.

26. Kehrer JP, Robertson JD, Smith CV. Free Radicals and Reactive Oxygen Species. In: McQueen CA, editor. Comprehensive Toxicology: Elsevier; 2010.

27. Khona DK, Shirolikar SM, Gawde KK, Hom E, Deodhar MA, D'Souza JS. Characterization of salt stress-induced palmelloids in the green alga, Chlamydomonas reinhardtii. Algal Research-Biomass Biofuels and Bioproducts. 2016; 16:434-48.

28. Khozin-Goldberg I, Shrestha P, Cohen Z. Mobilization of arachidonyl moieties from triacylglycerols into chloroplastic lipids following recovery from nitrogen starvation of the microalga Parietochloris incisa (vol 1738, pg 63, 2005). Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 2006;1761(4):501-.

29. Kil IS, Shin SW, Yeo HS, Lee YS, Park J-W. Mitochondrial NADP (+)-dependent isocitrate dehydrogenase protects cadmium-induced apoptosis. Molecular Pharmacology. 2006;70(3):1053-61.

30. Kosova K, Vitamvas P, Prasil IT, Renaut J. Plant proteome changes under abiotic

stress - Contribution of proteomics studies to understanding plant stress response. Journal of Proteomics. 2011;74(8):1301-22.

31. Lacerda CMR, Xin L, Rogers I, Reardon KF. Analysis of iTRAQ data using Mascot and Peaks quantification algorithms. Briefings in Functional Genomics & Proteomics. 2008;7(2):119-26.

32. Lee H, Nam K, Yang J-W, Han J-I, Chang YK. Synergistic interaction between metal ions in the sea salts and the extracellular polymeric substances for efficient microalgal harvesting. Algal Research-Biomass Biofuels and Bioproducts. 2016; 14:79-82.

33. Li FM, Hu HY. Isolation and characterization of a novel antialgal allelochemical from Phragmites communis. Applied and Environmental Microbiology. 2005;71(11):6545-53.

34. Li M, Gao L, Lin L. Specific growth rate, colonial morphology and extracellular polysaccharides (EPS) content of Scenedesmus obliquus grown under different levels of light limitation. Annales De Limnologie-International Journal of Limnology. 2015;51(4):329-34.

35. Livanos P, Apostolakos P, Galatis B. Plant cell division: ROS homeostasis is required. Plant signaling & behavior. 2012;7(7):771-8.

36. Longworth J. Proteomics in Microalgae: A Postgenomic Approach for Improved Biofuel Production. UK: The University of Sheffield; 2013.

37. Longworth J, Wu D, Huete-Ortega M, Wright PC, Vaidyanathan S. Proteome response of Phaeodactylum tricornutum, during lipid accumulation induced by

nitrogen depletion. Algal Research-Biomass Biofuels and Bioproducts. 2016; 18:213-24.

38. Lurling M. Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. Annales De Limnologie-International Journal of Limnology. 2003;39(2):85-101.

39. Lurling M, De Lange HJ, Van Donk E. Changes in food quality of the green alga Scenedesmus induced by Daphnia infochemicals: biochemical composition and morphology. Freshwater Biology. 1997;38(3):619-28.

40. Lürling M, Van Donk E. Grazer-induced colony formation in Scenedesmus: are there costs to being colonial? Oikos. 2000;88(1):111-8.

41. Michelet L, Roach T, Fischer BB, Bedhomme M, Lemaire SD, Krieger-Liszkay A. Down-regulation of catalase activity allows transient accumulation of a hydrogen peroxide signal in Chlamydomonas reinhardtii. Plant Cell and Environment. 2013;36(6):1204-13.

42. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends in Plant Science. 2011;16(6):300-9.

43. Montemezzani V, Duggan IC, Hogg ID, Craggs RJ. A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. Algal Research-Biomass Biofuels and Bioproducts. 2015; 11:211-26.

44. O'Donnell DR, Fey SB, Cottingham KL. Nutrient availability influences kairomone-induced defenses in Scenedesmus acutus (Chlorophyceae). Journal

of Plankton Research. 2013;35(1):191-200.

45. Oksanen J. Vegan: an introduction to ordination. 2017.

46. Pereira L. Porous graphitic carbon as a stationary phase in HPLC: Theory and applications. Journal of Liquid Chromatography & Related Technologies. 2008;31(11-12):1687-731.

47. Pillai S, Behra R, Nestler H, Suter MJF, Sigg L, Schirmer K. Linking toxicity and adaptive responses across the transcriptome, proteome, and phenotype of Chlamydomonas reinhardtii exposed to silver. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(9):3490-5.

48. Pitzschke A, Schikora A, Hirt H. MAPK cascade signalling networks in plant defence. Current Opinion in Plant Biology. 2009;12(4):421-6.

49. Pohnert G, Steinke M, Tollrian R. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends in Ecology & Evolution. 2007;22(4):198-204.

50. Poulson-Ellestad KL, Jones CM, Roy J, Viant MR, Fernandez FM, Kubanek J, et al. Metabolomics and proteomics reveal impacts of chemically mediated competition on marine plankton (vol 111, pg 9009, 2014). Proceedings of the National Academy of Sciences of the United States of America. 2014;111(34):12568-.

51. Prochnik SE, Umen J, Nedelcu AM, Hallmann A, Miller SM, Nishii I, et al. Genomic Analysis of Organismal Complexity in the Multicellular Green Alga Volvox carteri. Science. 2010;329(5988):223-6.

52. Rawlings AE. Membrane proteins: always an insoluble problem? Biochemical Society Transactions. 2016;44(3):790-5.

53. Roccuzzo S, Beckerman AP, Pandhal J. The use of natural infochemicals for sustainable and efficient harvesting of the microalgae Scenedesmus spp. for biotechnology: insights from a meta-analysis. Biotechnology Letters. 2016;38(12):1983-90.

54. Romero LC, Angeles Aroca M, Laureano-Marin AM, Moreno I, Garcia I, Gotor C. Cysteine and Cysteine-Related Signaling Pathways in Arabidopsis thaliana. Molecular Plant. 2014;7(2):264-76.

55. Russeth KP, Higgins L, Andrews MT. Identification of proteins from non-model organisms using mass spectrometry: Application to a hibernating mammal. Journal of Proteome Research. 2006;5(4):829-39.

56. Schey KL, Grey AC, Nicldayt JJ. Mass Spectrometry of Membrane Proteins: A Focus on Aquaporins. Biochemistry. 2013;52(22):3807-17.

57. Schmid J, Sieber V, Rehm B. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Frontiers in Microbiology. 2015;6.

58. Shanmuganathan A, Avery SV, Willetts SA, Houghton JE. Copper-induced oxidative stress in Saccharomyces cerevisiae targets enzymes of the glycolytic pathway. Febs Letters. 2004;556(1-3):253-9.

59. Shao J, Wu Z, Yu G, Peng X, Li R. Allelopathic mechanism of pyrogallol to Microcystis aeruginosa PCC7806 (Cyanobacteria): From views of gene expression and antioxidant system. Chemosphere. 2009;75(7):924-8.

60. Shi J, Chen Y, Xu Y, Ji D, Chen C, Xie C. Differential Proteomic Analysis by iTRAQ Reveals the Mechanism of Pyropia haitanensis Responding to High Temperature Stress. Scientific Reports. 2017;7.

61. Vallon O, Spalding MH. Organellar and Metabolic Processes. In: *Elizabeth H. Harris PD, David B. Stern, Ph.D., and George B. Witman, Ph.D.*, editor. The Chlamydomonas Sourcebook (Second Edition): Academic Press; 2009.

62. van Holthoon FL, van Beek TA, Lurling M, Van Donk E, De Groot A. Colony formation in Scenedesmus: a literature overview and further steps towards the chemical characterisation of the Daphnia kairomone. Hydrobiologia. 2003;491(1-3):241-54.

63. Verstrepen KJ, Klis FM. Flocculation, adhesion and biofilm formation in yeasts. Molecular Microbiology. 2006;60(1):5-15.

64. Wacker A, Piepho M, Harwood JL, Guschina IA, Arts MT. Light-Induced Changes in Fatty Acid Profiles of Specific Lipid Classes in Several Freshwater Phytoplankton Species. Frontiers in Plant Science. 2016;7.

65. Walley JW, Kliebenstein DJ, Bostock RM, Dehesh K. Fatty acids and early detection of pathogens. Current Opinion in Plant Biology. 2013;16(4):520-6.

66. Wang HP, Liu F, Luo P, Li ZH, Zheng LG, Wang H, et al. Allelopathic Effects of Myriophyllum aquaticum on Two Cyanobacteria of Anabaena flos-aquae and Microcystis aeruginosa. Bulletin of Environmental Contamination and Toxicology. 2017;98(4):556-61.

67. Wei S, Bian Y, Zhao Q, Chen S, Mao J, Song C, et al. Salinity-Induced Palmella

Formation Mechanism in Halotolerant Algae Dunaliella salina Revealed by Quantitative Proteomics and Phosphoproteomics. Frontiers in Plant Science. 2017;8.

68. Wilen B-M, Lumley D, Mattsson A, Mino T. Relationship between floc composition and flocculation and settling properties studied at a full scale activated sludge plant. Water Research. 2008;42(16):4404-18.

69. Wu X, Zhang J, Qin B, Cui G, Yang Z. Grazer density-dependent response of induced colony formation of Scenedesmus obliquus to grazing-associated infochemicals. Biochemical Systematics and Ecology. 2013; 50:286-92.

70. Xie G-J, Liu B-F, Xing D-F, Nan J, Ding J, Ren N-Q. Photo-fermentative bacteria aggregation triggered by L-cysteine during hydrogen production. Biotechnology for Biofuels. 2013;6.

71. Yang Y, Zheng N, Zhao X, Zhang Y, Han R, Ma L, et al. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. Journal of Proteomics. 2015; 116:34-43.

72. Yang Z, Kong F. Formation of large colonies: a defense mechanism of Microcystis aeruginosa under continuous grazing pressure by flagellate Ochromonas sp. Journal of Limnology. 2012;71(1):61-6.

73. Yang Z, Kong F, Shi X, Xing P, Zhang M. Effects of Daphnia-associated infochemicals on the morphology, polysaccharides content and PSII-Efficiency in Scenedesmus obliquus. International Review of Hydrobiology. 2007;92(6):618-+.

74. Yokoyama K, Saitoh S, Ishida M, Yamakawa Y, Nakamura K, Inoue, et al. Very-long-chain fatty acid-containing phospholipids accumulate in fatty acid synthase temperature-sensitive mutant strains of the fission yeast Schizosaccharomyces pombe fas2/lsd1. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2001;1532(3):223-33.

75. Yu W, Chen Z, Shen L, Wang Y, Li Q, Yan S, et al. Proteomic profiling of Bacillus licheniformis reveals a stress response mechanism in the synthesis of extracellular polymeric flocculants. Biotechnology and Bioengineering. 2016;113(4):797-806.

76. Zhang J, Xin L, Shan B, Chen W, Xie M, Yuen D, et al. PEAKS DB: De Novo Sequencing Assisted Database Search for Sensitive and Accurate Peptide Identification. Molecular & Cellular Proteomics. 2012;11(4).

77. Zhu XX, Yang JW, Xu N, Chen G, Yang Z. Combined effects of nitrogen levels and Daphnia culture filtrate on colony size of Scenedesmus obliquus. Algal Research-Biomass Biofuels and Bioproducts. 2015; 9:94-8.

78. Zhu X, Nan H, Chen Q, Wu Z, Wu X, Huang Y, et al. Potential grazing intensity directly determines the extent of grazer-induced colony formation in Scenedesmus obliquus. Biochemical Systematics and Ecology. 2015; 61:271-7.

79. Zhu X, Yang J, Zhang X, Zhang L, Wang X, Huang Y, et al. Low temperature and Daphnia-associated infochemicals promote colony formation of Scenedesmus obliquus and its harvesting. Biotechnology Letters. 2017;39(1):85-90.

SUPPORTING MATERIALS

SECTION I- IC, MC PEPTIDES INTENSITIES

iTRAQ#1		*Q (lin, MC)*							
	# uniq pepts	113	114	115	116	117	118	119	121
A0A097PB89	1	1	0.918686	0.930419	0.938899	1.073026	1.070706	1.058066	1.018892
A0A0C4K0H7;I0YIH9	2	1	0.95597	1.209563	1.140312	0.961315	1.020966	0.939085	0.924901
A0A172C1L3	1	1	0.994252	1.277696	1.037197	0.467412	0.886812	0.978037	1.142294
A0A172C1L3;Q1KVS9;A0A120N1C6;A0A172BZR9;A0A110B8L5;A0A0X8XG29;A0A110B817;A0A120N1C5;P17245;A0A097PBA2	1	1	1.01304	0.893112	0.923704	2.294774	0.959315	0.917171	0.895029
A8HW56;E1Z5R3;I0YZZ5	2	1	1.095212	0.946443	0.965885	0.748259	1.111164	1.272332	1.007438
A8HW56;I0YZZ5	1	1	0.912876	0.700446	0.757609	1.107076	0.813995	0.908053	0.894547
A8HYU5;C1N037	1	1	1.068775	1.083627	1.05078	0.920432	0.911484	1.120757	1.084179
A8IB25;D8TKV1	1	1	0.912316	0.81727	0.91631	0.939387	0.899878	0.870672	0.890123
A8IHL3;D8U3T1	1	1	1.022041	0.883651	0.857748	0.988684	1.030216	0.896556	0.905327
A8ILJ9;D8TPH9	1	1	1.021811	0.867201	0.855616	0.8807	0.755289	1.148642	1.252593

A8IRT2;I0YSF0;C1MLJ8;E1ZTE2;D8TV91	1	1	0.913846	1.098199	1.043521	1.485477	1.34568	1.251226	1.237282
A8ISB0;A8ISA9;D8TSY0;D8TK58;I0Z3J7	1	1	1.073804	2.152402	0.84095	0.98372	0.658099	0.599601	0.977259
A8IX80;D8UGB5	2	1	1.034744	1.274165	1.181027	1.057726	1.072263	1.103216	1.026486
A8IZU0;D8TMR1;B7TJI1;C1MVP3;D8UI03;E1ZE03;A8HYV3;Q8VY41;Q9M452;I0Z190;E1Z7R4;K8ENF9	1	1	1.013817	1.23126	1.241839	0.946853	1.250043	1.036302	1.045338
A8IZZ4;D8U995;D8U547;A8JF18_CHLREUbiquitin,minorisoformOS=Chlamydomonasreinhardtii GN=UBQ1a;A8JCX9;D8UEE9;A8JF17_CHLREBi-ubiquitin,majorisoformOS=Chlamydomonasreinhardtii GN=UBQ1a;I0YMQ7;D8U474;I0Z619;E1ZCE0;E1Z8A6;C1MSH9;A4S1B1;K8EHK7;A4RZS0;C1N2W9;K8F0B2;C1N647;K8EP62;K8F2N1;C1N	1	1	1.025557	1.287031	1.174678	1.245805	1.454301	1.228974	1.404601

1Q7;A4S5I2;E1ZK88;D8LC68;D7FWC9									
A8J6C7;D8TTK4;I0Z5Q8	2	1	0.985936	0.998529	0.99551	1.059677	0.996913	1.001575	1.079472
A8J979	1	1	0.974296	0.971346	0.992469	0.94599	0.975294	1.019308	1.031788
A8JCY4	4	1	0.957014	1.057881	1.063161	0.978867	1.161921	1.123637	0.925931
A8JDV2;D8UIE7	2	1	1.096387	0.974852	0.965198	1.314451	0.949118	1.059517	1.054224
A8JEU4;Q8RY44	1	1	1.05836	0.926474	0.989759	0.957707	1.079565	0.929459	0.768302
A8JHX9	2	1	0.990113	0.849237	1.006389	1.024481	1.027862	0.835435	0.97002
A8JID6;D8TLN9;E1ZPP6	1	1	1.06012	0.935911	0.957884	1.743998	1.471866	1.216459	1.107943
A8JJG8;A8JJV5;A4S1C9;K8EHQ7;C1MHL2;A8JJN6;D8UMG1;A8JJS0;A8IJR6;A8JIN6;A8JDH1;A8JDE1;A8JDC9;A8JDC0;A8IR79;A8IR69;A8IJS4;A8HWX5;A8HWX1;A8HWE3;A8HV98;D8TP10;D8TNF1;D8UDT7;A8IW84;A8IW75;D8U9Y1;D8TZB9;A8HSB2;D8TM85;D8TI76;D8TIA7;D8TI79;K8EFG9;K8EZ76	1	1	1.187097	1.153725	1.428805	1.73537	1.445133	1.221477	1.190205

B0JWT7	1	1	0.882087	1.029084	0.955422	1.028555	1.087236	1.061171	1.114758
B0JXA3	1	1	0.928753	1.189212	1.070349	0.992301	1.10915	1.077213	1.099299
B7TJI2	1	1	1.122072	1.357675	1.172562	0.371261	1.136044	1.179995	1.595898
C1MNA2;D8U0E5	1	1	0.913188	1.156672	1.269176	0.973676	1.098412	1.042383	1.112163
C1N789	2	1	0.947461	1.015948	1.042162	0.988309	0.975416	1.040142	1.069951
CON__P00761	4	1	1.006903	0.847683	0.949084	1.532383	1.168794	1.212015	1.395088
CON__P04264	2	1	0.995489	1.059066	1.050708	1.009116	1.032726	1.017929	1.14235
D8TJ31	1	1	0.910267	0.956986	0.946469	1.035361	0.835864	0.864274	0.875192
D8TPY4	1	1	0.789535	1.300561	1.976124	1.551024	2.574026	1.380433	1.279833
D8TT41;A8I7T8;A8I7S9;A4RSV4	1	1	1.068362	1.092118	0.991108	0.787961	0.932492	0.919517	0.990259
D8TUG4;A8J7H3	1	1	0.921469	0.669582	0.806226	0.843539	0.760351	0.845393	0.787506
D8TUP1	1	1	1.021525	1.219903	1.245973	0.73403	1.22972	0.754554	0.680608
D8TV46;A8IRQ1	1	1	1.03471	1.151311	1.038027	0.955289	0.925959	0.986248	1.042587
D8U1F3;A8IW39	2	1	1.015051	1.031179	1.122163	1.14975	1.059812	1.082377	1.04325
D8U1I3;I0YVA0	1	1	1.024546	0.855228	0.8774	1.032426	0.97894	1.03482	0.846848
D8U1R3;E1ZP98	1	1	1.028936	1.181244	0.973722	1.080306	1.033712	1.04546	1.193127
D8U1R3;E1ZP98;I0Z1A5;B0JJ69;	1	1	1.09287	1.392541	1.035516	1.344661	1.277086	1.428759	1.347069

C1N726;A4SAW5;A8JK20;K8F2G0									
D8U477;A8ILN4;D7FRY5;A4S2B3;C1MNJ9;K8EKA1;I0Z4W2	1	1	0.951343	1.115133	1.029464	1.058785	1.040722	1.024604	1.090224
D8U477;A8ILN4;D7FRY5;I0Z4W2	1	1	1.254925	0.881397	0.996602	0.996572	0.961736	1.092875	1.12339
D8U4Q1	2	1	1.073675	0.813095	0.866984	0.719731	0.876042	0.946684	0.949798
D8U5B1;A8JG03	5	1	1.020671	0.963536	0.983281	1.007166	1.007379	0.95701	0.933153
D8UC42;A8IA45;I0Z9U5	1	1	1.073964	1.131494	1.088494	0.710276	0.981277	0.92883	0.912501
D8UI03;E1ZE03;A8HYV3;Q8VY41;Q9M452;I0Z190	1	1	0.963358	0.72705	0.816882	1.089006	0.948518	0.908036	0.957299
E1Z5P4;A8JGF4	1	1	1.16335	1.120074	1.171077	1.314471	1.15156	1.304264	1.25595
E1Z746	1	1	1.072988	1.195143	1.020462	0.983246	1.218879	1.131584	1.017279
E1ZBK2	1	1	1.138877	0.398359	0.717128	1.330606	0.920715	0.997032	0.830023
E1ZBK2;D8TNN3;D8THW4;A8HX38	3	1	1.083179	0.653839	0.830838	1.434102	0.864082	1.019003	0.93931
E1ZBK2;D8TNN3;D8THW4;A8HX38;A4S6B6;K8F4B8;C1MZI5;C1MT59	1	1	0.977933	0.686195	0.771872	0.985397	0.896001	0.913684	0.857765

E1ZEB1;A8HXL8;D8TI16;I0ZA63	1	1	1.075616	1.108832	1.10104	1.105552	1.210888	0.896959	0.967025
E1ZFM2;A8I9H5;D8UIJ0	2	1	0.97121	1.219147	1.060649	0.864433	1.023583	0.954972	1.075206
E1ZJQ8	1	1	0.959116	0.907586	0.94511	1.143672	0.862696	0.901169	0.897068
E1ZQL8	1	1	1.049471	1.328948	0.98459	1.000938	1.082598	0.885357	0.952057
E1ZQY4	2	1	1.111618	0.974271	0.888935	1.12912	1.357361	1.262499	1.519427
E1ZSU0	1	1	0.922143	0.874261	0.827397	0.655168	0.704991	0.690725	0.742682
I0YP36	1	1	1.050704	0.8227	0.837918	0.546914	0.759242	1.033576	1.037686
I0YQ64;A8J537	1	1	1.085065	1.575704	1.370149	0.985257	1.391966	1.199622	1.219184
I0YRY7;Q56D00;E1ZIV3	1	1	0.934588	0.536065	0.760483	3.123741	0.675295	0.932524	0.81838
I0YS06;H2ELS9;D8TSK8;A8JHQ7;C1MIT8	1	1	0.908463	1.307878	1.125031	1.43314	1.16551	1.220943	1.39223
I0Z028	1	1	1.048408	1.382825	1.426081	1.021708	1.338776	1.162463	1.118502
I0Z3A2	2	1	0.894079	0.994981	0.940679	0.994811	0.834734	0.89784	0.994305
I0Z401	2	1	0.908304	0.9704	0.950469	0.861922	0.755882	0.776687	0.775113
I0Z6P1;A8HYD2	1	1	0.98195	0.802011	0.904102	1.250997	0.975046	1.02454	0.915931
I0Z918	1	1	0.956848	0.962323	0.862417	0.851362	0.848237	0.857628	0.985751
K8EQC7;C1MZG8;I0Z9Y9;D8TIF4	2	1	0.999144	0.803607	0.782331	1.681818	0.873172	0.893899	0.772716

K8F1S2	1	1	0.997229	1.111999	1.052046	0.931933	1.081616	1.037257	1.074468
K8F9G7	1	1	1.001527	1.08626	1.058801	0.892459	0.935661	0.754771	0.754512
P02769;CON__P02769	24	1	0.947209	0.988409	1.0146	1.288407	0.969206	1.127165	1.47956
P06007;Q1KVW6	1	1	0.875231	1.11289	1.109613	0.853725	0.994839	0.978263	0.990758
P06007;Q1KVW6;Q4JLT1;K8FE34;K7NRG3;F2YGQ0;E9NPS3;D0FXW8;A0A1C8XRK9;D1J6Z4;B0JR69;P48079;A0A097PB60	1	1	0.940605	1.253914	1.27132	1.132459	1.327675	1.1345	1.175514
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3;Q1KVU0;B2LWG0;D8UK13;Q8SLI8	1	1	0.958913	0.938956	0.889983	0.999063	1.008377	0.939964	0.956862
Q00914;K7NRF9;D0FXW7;D1J7C7	1	1	1.132223	1.091791	1.046607	1.263409	1.132164	1.559834	1.426462
Q1KVT0	2	1	0.961667	1.169059	1.101292	0.9521	1.056173	0.881818	0.966392
Q1KVT0;P06541;D0FXY0;A0A1C8XRG2;Q8HDD9;K7NVH0;K8FHJ4;Q8HDG4	1	1	0.925457	1.010243	0.94691	0.717526	1.010618	0.956658	0.719944
Q1KVT0;P06541;D0FXY0;A0A1C	2	1	0.883388	0.931884	0.936692	0.858041	1.024234	0.934054	1.061947

8XRG2;Q8HDD9;K7NVH0;Q8HDG4									
Q1KVT0;P06541;P48081;A0A097PBH6;Q8HDG4	1	1	0.888296	0.612675	0.638781	0.831878	0.708125	0.960012	1.139208
Q1KVT0;P06541;Q8HDG4	3	1	1.060718	1.599139	1.246143	0.592354	1.062986	1.148888	1.394617
Q1KVU8;F2YGK0	1	1	0.812787	0.893365	1.065436	1.544234	1.298727	1.092696	1.111981
Q1KVV6	1	1	0.884502	0.964271	1.240556	2.1739	1.384237	1.470909	1.575521
Q1KVY1	2	1	1.006973	1.035984	1.001681	1.20716	1.166539	1.065365	1.167906
Q42690	1	1	1.164257	0.742386	0.809863	1.090678	0.833923	1.089622	0.96172
Q42690;D8TKY4;I0YN66;E1ZQQ5	2	1	0.944723	1.220798	1.151657	0.913439	1.145052	0.958495	0.936729
Q84RL9	2	1	0.982926	1.197091	1.145182	0.923573	1.192973	1.029342	1.052124
Q8HDG4	1	1	0.968624	1.347793	1.236989	0.929775	1.177837	0.897366	0.926961
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;A0A0X9AMW9;A0A0X9AGK8;E9NPX3;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;A0A172C918	2	1	1.012231	0.680534	0.857593	0.998881	0.935107	0.805028	0.744844

S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;A0A0X9AMW9;A0A0X9AGK8;E9NPX3;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;W6A299;Q1KVV0;F2YGL1;A0A172C918;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;	1	1	0.940029	0.772272	0.917402	1.905976	1.125131	1.035692	0.87862

Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;P00877;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1VNR7;M1VK51;M1VK44;M1VEJ4;M1V8T0;M1VEI8;Q3S3F0;Q2I3J7;Q8HD99;W6A241									
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;A0A0X9AMW9;A0A0X9AGK8;E9NPX3;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;W6A299;Q1KVV0;F2YGL1;Q8HD99;W6A241	4	1	1.070202	0.869989	0.973069	0.404575	0.949408	0.946723	0.910442
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8	1	1	1.011844	0.96142	1.028464	0.919092	0.982334	0.895325	0.850462

J6;A0A0X8XG25;W6AAY4;W6AAZ3;E9NPX3;W6A299;Q1KVV0;F2YGL1;A0A172C918;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TG

Z2;P00877;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1V8T0;M1UZB8;M1VEI8;Q2I3J7;W6A1S2;A0A1S6M237;P24312;A0A023SZZ9;A0A0A0Y7C9;A0A140CQM1;R4ITL5;A0A140CQM0;Q8HD99;W6A241									
A0A097PB89;D1J797;B0JWV1;P48080;E9NPZ5;D8LJM3;P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3;Q1KVU0;F2YGQ9	1	1	0.927625	1.053814	1.05516	1.021162	0.890921	0.905357	1.040981
A4S0V1	1	1	0.936274	1.310767	1.265296	0.82955	1.22464	1.043481	1.024982
A4S824;D8UF17;A8IWK2;K8F1R7;C1MYV2	1	1	1.005871	0.81488	1.004198	1.048871	1.141261	1.19353	1.001298
A8IQU3;D8TRA2	5	1	0.916466	0.86169	0.864882	1.051163	0.930203	1.040848	1.023276
A8IQU3;D8TRA2;E1ZS63;I0YLJ1	1	1	1.052823	0.763898	0.752832	0.934311	0.728383	0.923776	0.931786
A8IWQ7;D8UEY8	2	1	1.029078	1.199274	0.967028	0.838852	1.085463	1.167816	1.266955
A8IY43;D8U4U4	1	1	1.116267	1.303188	1.050176	0.461926	0.998477	1.04166	1.323562
A8IYP4;D8TRR7;	1	1	0.894792	0.937308	1.010527	1.951929	1.074616	0.869956	0.850433

E1ZF27									
A8IZU0;D8TMR1	2	1	1.02092	0.868368	0.95981	1.103474	1.051777	1.023933	1.075614
A8IZZ4;D8U995;D8U547;A8JF18_CHLREUbiquitin,minorisoformOS=Chlamydomonasreinhardtii GN=UBQ1a;A8JCX9;D8UEE9;A8JF17_CHLREBi-ubiquitin,majorisoformOS=Chlamydomonasreinhardtii GN=UBQ1a;I0YMQ7;D8U474;I0Z619;E1ZCE0;E1Z8A6;E1ZHZ0	1	1	1.009599	1.013069	1.00029	1.938178	1.038717	1.034563	1.158807
A8J6K9	1	1	1.126316	1.012228	1.024103	1.237322	1.020391	1.171733	1.170561
A8JEU4;Q8RY44;E1ZQV2	1	1	1.041753	1.077429	1.289142	0.82805	0.926725	0.994909	1.134735
A8JHB4;B0JJU1	1	1	1.013777	1.10429	1.491301	0.987943	1.439082	0.843381	0.708596
A8JHB4;D8TNQ3	1	1	0.892847	1.014148	0.995848	0.820857	0.900134	0.794807	0.860605
C1MHD4;E1ZGF5	1	1	0.891293	0.858314	0.845237	1.029146	0.948969	0.959551	0.934208
C1MJ78	1	1	0.914315	0.715082	1.029499	0.535592	1.277314	0.560652	0.654831
C1ML07	1	1	0.928566	1.049341	1.005781	1.010068	0.923283	0.94304	0.985962

C1MU18;A4RYP4	1	1	1.036473	0.978965	0.995928	0.94292	0.898502	1.003523	0.933789
C1MYV3;E1ZLQ3;I0YI95;D8UA08;A8JAV1;O03989;D7FQK6;Q9SWF3	1	1	0.8694	1.131517	0.997204	0.823322	0.906654	0.89546	0.866364
C1N5G3	1	1	1.013044	0.953149	1.088076	1.162386	1.150982	1.096256	1.160087
CON__P13717	4	1	0.862855	0.901209	0.83152	1.210811	1.051966	1.055093	1.404477
D7FK90;D8LI58;D7FZN2;E1ZQV2	1	1	1.005636	1.209139	1.188694	0.960191	1.130076	0.989878	0.887168
D8TJU4	1	1	0.9308	1.15298	1.083797	1.118162	0.925253	1.135231	1.132373
D8TPD5;A8IL08;I0Z5A8	2	1	1.006166	1.043619	1.058561	1.207458	1.045522	1.079811	1.101825
D8TQM8;A8J3Y6	1	1	0.898381	0.972807	0.921015	0.802713	0.878493	0.895133	0.865265
D8TTA3	5	1	0.992582	1.007471	0.958455	0.975671	0.935269	0.970952	1.000739
D8TV46;A8IRQ1;E1Z7C4	2	1	1.034354	1.084902	0.998025	0.870374	0.928422	1.014042	1.012129
D8TW10;E1ZKW6	2	1	1.065382	1.102101	1.003998	0.988402	0.818018	0.959741	0.875354
D8U1R3	2	1	1.004581	0.93264	1.0218	1.214504	1.15472	1.011884	1.013124
D8U1R3;E1ZP98;I0Z1A5;B0JJ69	2	1	1.004475	1.158047	1.144157	0.863654	1.205779	1.019907	1.084982
D8U973;A8IZW	1	1	1.017484	1.104788	1.060235	0.86764	1.076566	1.035632	0.956918

6									
D8U992	1	1	1.108792	1.248332	1.241493	1.173785	1.21509	1.135197	1.513966
D8U992;Q9ZTA7	1	1	0.966989	0.800959	0.846208	0.883176	0.877908	0.904722	0.922752
D8UBA1	1	1	0.900752	0.970121	1.042394	0.955677	0.847828	0.903825	0.962042
D8UDE0;A8HPL8;E1ZJ54	1	1	0.886354	0.897786	0.764119	0.655885	0.649059	0.78821	0.736259
D8UE23;A8IVM9;E1Z7V9	1	1	0.988404	0.957392	0.941218	0.92515	0.93807	1.218481	1.101615
D8UF03	2	1	0.93428	1.025816	0.934826	1.038669	0.966964	1.017727	0.951347
D8UFR3;A8J9T0	3	1	1.152281	0.819209	0.787582	1.214088	0.952467	1.102025	1.066349
E1Z356	1	1	1.059449	1.117597	0.997616	1.052733	1.106228	1.123505	1.14347
E1Z6L2	2	1	1.02542	1.114104	0.955137	0.94245	0.905721	1.016429	1.0371
E1Z7C4	1	1	0.969715	0.955863	0.937646	0.87561	0.775547	0.976778	1.044846
E1ZFQ1	1	1	1.027023	0.873838	0.840255	1.200626	0.727653	0.929513	0.859527
E1ZMW8	1	1	1.023496	0.907058	1.029766	1.176657	0.955988	1.121315	1.02765
I0YPF7;A8IP17;D8TY33	1	1	0.962716	0.727397	0.889162	0.971506	1.064092	0.920299	0.919432
I0YQW6;D4N535	1	1	0.900426	0.821171	0.771281	0.765761	1.003993	0.976503	0.999262
I0YV40	1	1	0.92051	1.083631	0.88732	0.855415	1.193251	1.046368	0.98504
I0YZ27	1	1	1.020505	1.260813	1.197567	1.024793	1.043969	1.06835	1.175453

K8EHR6;A4S7X2;C1N6J0	1	1	0.824571	1.174113	1.511792	2.016667	1.630156	1.490693	1.682647
Q1KVT0;Q8HDG4	2	1	0.908251	0.752388	0.767335	2.083475	0.746766	0.88644	0.900079
Q1KVU3	2	1	0.872288	0.782641	0.949861	1.588874	0.814265	0.869624	0.849477
Q1KVX3;K7NSN1;A0A1C8XRP4	2	1	0.951153	0.983026	1.033415	1.343511	1.103185	1.070187	1.147636
Q1KVY2	2	1	0.949637	1.295047	1.278343	0.916305	1.373959	0.912463	0.982037
Q6J213;I0YWB9	1	1	1.035518	0.846193	0.81787	1.09626	0.917703	0.998427	1.117444
Q763T6	1	1	0.953942	1.083805	1.032194	0.968124	0.902915	0.97656	1.040638
Q8HDD7	1	1	0.803065	0.979174	0.928113	0.765678	0.907296	1.029241	0.978192
Q8VXQ9	2	1	1.290262	2.119706	1.625935	0.469618	1.260912	1.106407	1.204662
Q9FE86	1	1	1.041305	0.902923	0.992771	1.510348	0.991736	1.016259	0.932646
S4ULQ5	1	1	1.067211	1.302713	0.914965	0.672059	0.811899	0.711121	0.700771
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V	1	1	1.124784	0.737211	0.842099	0.893195	0.862485	0.790461	0.682155

743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;P00877;K7NSN7;D0FXZ7;A0A1C8XRQ3;M1VNR7;M1VK51;M1VK44;M1VEJ4;M1V8T0;M1UZB8;Q3S3F0;W6A1S2;Q319

12;P24312;A0A023SZZ9;A0A023T0H1;Q8HD99;W6A241;A0A023SYL4									
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;E9NPX3;W6A299;Q1KVV0;F2YGL1;A0A172C918;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L	1	1	1.030767	0.74311	0.752929	1.96199	0.755931	0.969661	0.789603

2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;P00877;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1V8T0;M1UZB8;M1VEI8;Q2I3J7;W6A1S2;A0A1S6M237;P24312;A0A023SZZ9;A0A0A0Y7C9;A0A140CQM1;R4ITL5;A0A140CQM0;S4VV39;Q8HD99;W6A241									
A0A0S1LH61	1	1	1.037601	1.259927	1.219844	0.997411	1.143499	1.091649	0.991194
A0A172C1L3;Q1KVS9;A0A120N1C6;A0A172BZR9;A0A110B8L5;A0A0X8XG29;P17746;K7NSQ0;D0FXV6;A0A1C8XRX1;A0A110B817;A0A120N1C5	1	1	1.033752	0.856794	0.899288	1.064728	0.890417	0.93726	0.913588
A0A172C1L3;Q1KVS9;A0A172BZ	1	1	1.036397	1.072677	1.026578	0.953974	0.990517	1.033981	1.033309

R9;A0A0X8XG29;K7NSQ0;D0FXV6;A0A1C8XRX1;A0A120N1C5									
A4RTP0	1	1	1.095462	1.139009	1.294549	1.060677	1.368522	1.087555	1.133929
A4RTP0;C1MJJ1;D8U848;A8ICG9;E1Z349;I0Z036	1	1	1.031779	1.258709	1.138463	0.890451	1.084755	1.064855	1.073894
A4S614;C1MJ74	1	1	1.02622	1.012754	0.887791	0.952226	1.034779	1.053858	1.191171
A8IMY5	1	1	0.866359	0.818238	0.828565	0.924764	0.850561	0.865826	0.903448
A8IQU3;D8TRA2;K8EN95;A4RSS5;C1MKD5	1	1	0.999839	1.083812	1.077685	1.086282	1.20695	1.037169	1.046112
A8IQU3;D8TRA2;K8EN95;A4RSS5;C1MKD5;I0YLJ1	1	1	1.000443	0.5764	0.71058	0.92168	0.612782	0.979636	1.049764
A8IRT2	1	1	1.066397	1.316198	1.144238	1.276644	1.311214	1.230313	1.174814
A8IZU0;D8TMR1;A4RWG3;K8F7J7;C1MP69	1	1	1.071697	1.073858	1.159048	1.004828	1.064083	1.047132	1.066739
A8J1M9;D8TL63	1	1	1.038968	1.158621	1.080762	0.989552	1.057203	0.991573	0.989815
A8J237	1	1	1.053396	1.161365	1.265975	1.234582	1.704381	0.791806	1.251773
A8J6K9;D8THE2	1	1	1.382198	1.07719	1.016637	1.266676	0.956411	1.150694	1.343839
A8JFI7;K8EU82;A4S0L7;C1MZE1	1	1	1.031678	0.877488	0.810957	0.804986	0.629214	0.919893	0.860989

;E1Z2Y2;D8UC03;I0Z0U2									
C1MU18;K8EF58;I0YNY5	1	1	0.874786	1.006701	0.827874	1.79913	1.238549	0.925703	1.075779
C1MYV3;E1ZLQ3;I0YI95;D8UA08;A8JAV1;Q9SWF3	1	1	1.338794	1.419372	0.883367	0.980962	0.736611	0.801222	0.766239
D8TK12	1	1	0.931549	0.60272	0.622444	1.308593	0.664479	0.850418	0.914583
D8TLB0;A8J1U1;Q002K0;Q002J6;Q002K1;Q002J7;Q002K2;Q002J8;Q002K3;Q002J9;A7M6Q3;I3RV97;I3RV96;A4S3L2;C1MQC4;I0YJJ0;K8EKU3	1	1	1.00505	1.015866	1.301394	0.981504	1.323662	0.983435	0.783597
D8TTF7	1	1	1.052587	0.955372	0.838825	0.965904	0.863525	1.08365	1.085053
D8TWH5;A8JFB1	1	1	1.072155	0.886242	0.969845	0.963414	0.906442	0.943538	0.981477
D8TWH5;E1ZRQ7	1	1	0.914142	0.989013	1.058324	0.765334	0.980528	0.806651	0.685886
D8TZD7;A8ITH8;I0YWY2;E1ZRQ6;K8EB57	1	1	0.976642	1.299965	1.248743	1.022818	1.267049	1.130866	1.121378
D8U4B4;A8J597	1	1	0.869236	0.966076	0.965725	1.071968	0.918758	0.854363	0.918121
D8U4Q1;E1ZGR1;A8IAN1;K8ERB6;I0YJZ4	1	1	1.046166	0.453909	0.490625	1.263248	0.668515	0.842612	0.736737

D8U6E0;A8I604	1	1	0.912325	1.126592	1.071493	1.033392	1.451515	1.238971	1.1387
D8UE23	2	1	1.074197	1.161846	1.04179	1.061821	1.111323	1.015712	0.950196
D8UFZ3;E1Z4A2;A8J9S7	1	1	0.931801	0.812241	0.997617	0.795268	0.949235	0.809447	0.8386
D8UI03;E1ZE03;A8HYV3	1	1	1.072271	1.02068	1.021307	1.761815	1.00534	0.907976	0.931918
D8UI03;E1ZE03;A8HYV3;Q8VY41;Q9M452	2	1	1.018808	1.182651	1.12211	1.079849	1.270218	1.117122	1.145637
E1Z298;D8TWN7;D8LKH8	2	1	1.009456	1.24587	1.188151	0.613816	1.157991	1.078962	1.130798
E9NPW9	1	1	0.994733	1.135662	1.154478	0.951806	1.077118	0.953537	0.9204
I0YL77;E1ZL24;D8TPC8;A8IL29;A4S5Z2;C1MH11;K8FE75	1	1	1.190545	0.967196	1.285057	0.581133	1.489519	1.251673	1.337879
I0Z4W2	1	1	1.006719	0.905666	0.821541	0.895226	0.9055	0.889267	1.083672
K8F0N5	1	1	0.946428	1.0116	0.876104	0.937239	0.807366	0.956923	0.920454
P26526;B7U1J0	3	1	0.933874	1.128223	1.047	0.960408	1.120355	0.923155	0.961452
Q1KVS9;P17746;K7NSQ0;D0FXV6;A0A1C8XRX1	1	1	0.988548	1.201633	1.181078	0.983166	1.105652	1.051797	1.113098
Q1KVT2;D8UMA6;Q00471	1	1	0.884472	0.75917	0.985295	2.634648	1.30233	0.938933	0.978481
Q1KVV6;Q2TGZ	1	1	0.87594	1.615931	1.865152	1.341308	1.833599	1.612529	1.720388

4;D0FY05;A0A1C8XRM6;P37255									
Q6J213;D8TP57	1	1	0.950895	0.907925	0.897035	0.957746	0.974684	0.955663	0.98352
Q763T6;E1ZRI5;D8U7C0;I0YKU6	1	1	0.954427	1.062899	0.978957	0.90841	1.003677	0.9557	0.95581
Q8LRU1;I0YP34;D8TX08	1	1	0.960875	0.829674	0.903811	2.559959	0.863659	0.850178	0.872696
Q9FEK6	1	1	0.955268	1.000747	1.2267	1.318026	1.125857	1.263041	1.14313
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;A0A0X9AMW9;A0A0X9AGK8;A0A172C918	1	1	1.43732	2.105801	1.613548	0.647599	1.071633	1.185214	1.310708
A0A1B0VE51	1	1	0.933655	1.194828	1.173781	1.12781	1.163659	1.026343	1.140048
A8HNE8;D8UHM8	1	1	0.930851	0.960512	0.936409	0.907668	0.857249	0.840941	0.748741
A8HRP1;D8TM86	1	1	0.887251	0.657205	0.911352	1.03464	0.837159	0.932632	0.839209
A8HZZ1;D8TM26;I0YN25;E1ZCK4	1	1	0.953409	1.084424	1.078349	0.944465	1.055761	1.006598	1.043894
A8I8Z4	1	1	0.856231	1.151797	1.031667	0.846526	0.912715	0.828701	0.935718
A8J5P7;D8TNA2	1	1	0.937271	1.143337	1.006312	1.089511	1.021122	1.117786	1.117648

A8JCY4;D8U593;I0YSE8	2	1	0.963889	1.203787	1.159949	0.898017	1.16768	0.809714	0.775283
A8JEU4;A4RSP0;Q8RY44;E1ZQV2	1	1	0.969784	1.139394	1.18247	0.991732	1.065765	0.833848	0.992525
A8JEU4;E1ZQV2	1	1	0.961053	0.504931	0.597085	0.917918	0.743183	0.732573	0.620718
D7G034;I0YMX2;E1ZNM7;A8J8B3;K8F1Y0	1	1	0.996944	0.979677	0.937215	0.989311	0.927405	1.1004	1.03576
D8TN65;A8IJ19	3	1	1.023265	1.122164	1.042017	0.931007	1.000239	1.012579	0.965749
D8TP83;I0YQQ4;A8IKP1;E1ZQ26	1	1	0.742703	0.92544	0.879438	1.124065	0.816873	0.907611	1.024114
D8TUP1;A8J7F6	1	1	0.95219	0.866196	0.946957	0.893298	0.855121	0.814702	0.783426
D8TZU3;A4RW20;E1Z378;K8F6A2	1	1	1.013831	0.835672	0.918785	1.19824	0.907285	0.924186	0.830399
D8U0Q5	1	1	1.095693	0.645871	0.853993	1.370024	0.962949	1.031356	0.796539
D8UF20;A8IWJ5;E1ZAJ1	1	1	0.856342	0.63339	0.82679	1.560726	0.973781	0.794668	0.800595
E1ZM20	2	1	0.935582	0.865321	0.793118	0.824272	0.878615	0.964851	1.031559
E1ZQL8;K8EP91;D8TUG4	1	1	0.786515	0.892208	0.885957	0.813934	0.880681	0.917409	0.736318
E1ZRA9	1	1	0.864607	1.007536	0.938372	0.916118	0.971715	1.146762	0.756503
E1ZT16	1	1	0.945919	0.997095	0.868399	1.171298	1.14519	1.134434	1.153045

I0YRY7;Q56D00	1	1	0.969414	0.99454	1.011726	0.914529	0.945457	0.905462	0.926149
I0YTX9	1	1	0.94748	0.982041	0.857289	1.085472	0.865018	0.907982	0.897329
I0YUW3	1	1	0.985667	0.815832	1.08454	1.32866	0.992023	1.343053	1.216457
I0YZE5	1	1	0.984162	0.933888	0.95921	1.729434	0.970837	0.99326	0.929165
I0YZE5;C1ML90;A4RRH9;A8IDP6;Q39708;D8TKN5;K8ENP9	1	1	0.96548	0.875557	1.03034	1.138273	1.037663	0.949273	0.915572
I0YZZ5	1	1	0.887062	0.783943	0.834895	0.980426	0.796639	1.01848	1.030569
I0Z4M6	1	1	1.017103	1.062044	1.01166	1.052647	0.961986	1.036475	0.99625
I0Z849	2	1	0.94083	0.95578	0.98094	1.072765	0.936848	0.91236	0.920042
K8ENF9	1	1	1.036075	1.027878	1.010138	0.958835	1.01716	1.001911	1.149265
Q1KVS9	2	1	1.01753	1.021643	0.962983	1.273517	0.947035	1.067686	1.08178
Q1KVS9;P17746;K7NSQ0;D0FXV6;A0A1C8XRX1;F2YGM8;C1KRB3;P17245;A0A097PBA2;K8F1E5;A8HXR2;A4RY66;D1J725;B0JSE0;E1Z696;I0YY77;C1MM21;I0YKL3;D8UI05;K8EC20;E9NPW9;D8LDT2	1	1	0.958646	0.8983	0.908213	0.952993	0.831469	0.897905	0.847975
Q1KVT0;P06541	1	1	0.960362	0.960778	0.887817	0.897373	0.872545	0.904507	0.980181

;D0FXY0;A0A1C8XRG2;Q8HDD9;K7NVH0;K8FHJ4;F2YGR0;E9NPS5;P48081;A0A097PBH6;D1J7B4;Q8HDG4									
Q1KVT2;E9NPX5;D1J798	1	1	1.016506	0.698427	0.999796	2.536879	1.517909	1.058038	0.981913
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3;A0A1C8XRL7;W8E1S1;B0JR68;F2YGQ1	1	1	1.00503	1.123207	1.297762	1.402792	1.363509	1.168284	1.259459
A8HNE8;D8UHM8;C1MVN3;E1Z4F7;B0JWT7;I0YIF2	1	1	0.993424	1.155016	1.066215	1.260304	1.102871	1.028114	1.062947
A8HS14;E1ZTI5;D8TZQ2;I0Z1V7	1	1	1.030648	0.925344	1.168276	0.941645	0.906161	0.994852	1.179673
A8IMK1;C1NAA3;D8TKA7	2	1	1.031551	1.143618	1.10153	0.769428	0.842773	0.95836	0.979394
A8IZU0	1	1	1.036625	0.936384	1.053323	1.046037	1.129419	1.201062	1.370113
A8J1G8	1	1	0.947091	0.990249	0.987599	1.3964	1.189197	1.190636	1.107578
A8JBG5	1	1	1.158923	1.846472	1.712489	0.540602	1.374854	1.154727	1.220362
A8JDW2;D8U3S7	1	1	1.033961	0.875586	0.889499	0.884406	0.84046	0.969699	0.96606
A8JFZ0_CHLRESerineglyoxylate	1	1	1.062431	0.974782	0.804588	1.15775	1.143558	1.122416	1.201166

aminotransferaseOS=Chlamydomonasreinhardtii GN=SGA1a;A8JFY9_CHLRESerineglyoxylateaminotransferaseOS=Chlamydomonasreinhardtii GN=SGA1a;D8U556									
A8JHB4	1	1	0.853677	1.350669	1.298568	0.705234	1.093215	0.736643	0.677419
B6E5W6;I0Z5K3	1	1	0.984342	1.055331	1.020243	1.092593	0.958307	0.96815	0.96372
C1MYV3;E1ZLQ3	1	1	1.085546	0.94824	1.034548	1.080193	0.945686	0.919102	1.02996
C1N9S5	1	1	0.995353	0.961262	1.187109	0.839821	1.111033	1.219272	1.100873
D8THK6;A8HXS9	1	1	0.92486	1.023342	0.921303	0.912594	0.883231	0.926247	1.054557
D8TJY9;A8IRK4	1	1	1.21578	0.900999	0.697992	1.816212	0.727857	0.982227	0.913809
D8TNU3	1	1	1.066571	0.763718	0.910057	1.301701	1.065934	1.179988	1.049053
D8TPD5;A8IL08;K8EJA2	1	1	1.072612	1.068398	1.13266	1.036551	1.100002	0.980013	0.993068
D8TT41;A8I7T8;A8I7S9	1	1	0.943571	1.082083	0.902522	1.103429	0.894626	1.03229	1.072447
D8TUW7;A8IAT4;I0YXF1;C1N3E5;E1ZG55	1	1	0.99511	1.166798	1.119987	1.025046	1.084389	0.984387	1.028094
D8TV46	1	1	1.101214	1.827624	1.346708	0.396391	1.040917	1.115461	1.392941

D8U1T0	1	1	0.995881	0.999254	0.850499	0.871692	0.899732	1.106935	1.050084
D8U3K8;Q5NKW4	1	1	0.876786	1.020048	1.081436	1.378614	1.206692	1.287685	1.297281
D8UC42;A8IA45;K8EK64;A4S3H0	1	1	1.156743	0.926624	0.917464	1.156231	0.828789	1.106405	1.270437
D8UC42;A8IA45;K8EK64;I0Z9U5	1	1	1.156231	0.834592	0.851637	0.989595	0.675622	1.101081	1.127906
D8UEA2;A8JFV6	1	1	1.113892	0.886336	1.05446	1.106598	1.037376	1.015969	1.295686
E1Z7R4	1	1	1.012431	0.872725	1.111506	0.923513	1.274424	1.140298	0.804139
E1ZD58;I0YR87	1	1	0.935054	1.142086	1.084271	1.25532	1.006494	1.156642	1.198896
I0YRR8	1	1	0.879853	1.245697	1.032007	1.075953	1.01651	0.957837	1.119357
I0Z401;E1Z7W6;D8LB71;A8JHB4;B0JJU1	1	1	0.901038	0.63371	0.832396	0.99808	0.843615	0.974467	0.778967
K4EKL3	1	1	0.945423	0.85796	0.934329	1.205965	1.02321	1.093144	0.99162
K8EQC7;C1MZG8;G4WUV8;G3LTV5;A8J8Y1;A4S6H8	1	1	0.429895	0.54808	0.449734	0.617672	0.40143	0.481278	0.549814
P06007;Q1KVW6;Q4JLT1;K8FE34;K7NRG3;F2YGQ0;E9NPS3;D0FXW8;A0A1C8XRK9	1	1	0.915154	0.995308	1.057544	1.703624	1.169474	1.090001	1.090203

P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3	1	1	1.073003	1.0069	0.954383	1.030662	0.7977	0.92159	0.889883
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;Q1KVU0;F2YGQ9	1	1	0.90284	0.879972	0.782431	1.036643	1.01213	1.050946	1.025702
Q8VXQ9;Q1HVA2;E1ZT20;D8U9J4;A8HP84;Q1HVA0;B1PL92;I0YMA8	1	1	1.040652	1.69051	1.402251	0.858268	1.324958	0.985918	0.999063
A0A0C4K0H7	1	1	0.979116	1.047837	0.810423	1.009021	0.824305	0.914242	0.889171
A4RQS5;C1MLH6	1	1	0.874681	0.782253	0.826046	0.851487	0.728379	0.827166	0.941107
A4SB22	1	1	1.181653	1.193144	1.004747	0.714028	1.042173	1.059907	1.040587
A4SB22;K8EL02;C1MWS0;B5A517	1	1	0.934582	0.788901	0.796576	1.291738	0.991807	0.926573	0.863095
A8HW56;D8TIS4;E1Z5R3;C1MLD8;A4RRG4;K8E910;I0YZZ5	1	1	0.915689	0.857152	0.963972	1.039449	0.894918	0.89413	0.801938
A8IN95;D8TLU2	2	1	1.092077	1.245296	1.048004	1.022064	0.986095	1.092826	1.273496
A8ISB0;A8ISA9;D8TSY0;D8TK58;E1ZQX7;A8IEE5	1	1	1.030141	0.932976	0.887425	1.412025	0.897096	1.049289	1.0592
A8IVJ7	2	1	0.691311	0.854566	0.746408	0.743247	0.803597	0.665651	0.708304

A8IW00;D8TM93;A8IVZ9;D8TM95;I0YYN3	1	1	0.924217	1.100839	1.194097	0.907241	0.999761	0.896694	0.932243
A8IXE0;E1ZSI5;I0YKP7	1	1	1.007789	0.870206	1.005521	1.216506	1.038783	1.060546	1.025349
A8J680;D8TNW2;A8J682	1	1	1.110434	0.923244	0.826892	1.045459	0.923668	0.89686	0.880999
A8J841_CHLREHydroxymethylpyrimidinephosphatesynthaseOS=ChlamydomonasreinhardtiiGN=THICb;D8U387	1	1	1.001896	0.862923	1.004865	1.030332	0.782928	0.863595	0.817112
A8J906;D8TIJ1	1	1	1.136698	0.882182	0.910364	0.871704	0.900847	1.069593	1.042769
A8JFB1	1	1	1.000161	1.367103	1.162843	0.5715	0.83499	0.89947	0.910851
D7FK90;D8LI58	1	1	0.942684	0.719818	0.929556	1.207989	0.94073	0.767426	0.683788
D7G599	1	1	1.046251	0.971277	0.912442	0.848538	0.887331	0.829745	0.742451
D8TIF4	1	1	1.051415	0.904131	0.863585	1.367512	0.790433	1.026027	0.996457
D8TNE6	1	1	0.948866	1.041307	0.992168	0.992482	0.869078	0.993233	0.916259
D8TPM9;A8ICT1	1	1	0.931293	0.799955	0.847541	1.225191	0.785836	0.793423	0.786112
D8TYV7	1	1	1.031775	0.849118	0.895196	1.061778	1.038459	0.931166	0.84115
D8TZZ8;A8JIB7;K8FER3	1	1	1.200975	1.065708	0.915953	1.105026	1.087935	1.174305	1.217677
D8UBP2	1	1	1.089626	1.148649	1.170167	0.89494	1.070926	0.930291	0.87943

E1Z349;I0Z036	1	1	0.881702	0.980222	0.992649	1.033774	1.00029	1.024659	1.11658
E1ZCK4	1	1	0.960489	1.086497	1.097359	0.819897	0.963855	1.165862	1.104013
E1ZFD0	1	1	1.060408	0.741337	0.909407	1.097706	0.768482	0.980243	0.929246
E1ZI27;I0YWG6	1	1	0.9735	0.873888	0.991683	0.958664	0.884557	0.801127	0.889038
I0YLA9	1	1	0.930414	1.130684	1.045854	1.021813	1.127498	1.135411	1.133381
I0YNP6	1	1	0.906556	0.860214	1.000204	0.94367	0.922424	1.154089	1.01299
I0YRY7	1	1	1.105734	1.204065	1.213474	1.152286	1.248901	1.013256	1.288006
I0Z0B3	1	1	1.047762	1.135526	1.203475	0.963897	1.185739	1.121652	1.242654
I0Z1E7	1	1	1.01792	1.013579	1.019528	1.051226	0.934954	0.993586	0.896988
I0Z9U5;E1ZH03	1	1	1.107171	0.941902	1.107456	1.049052	1.047338	1.004378	1.147822
K8EDQ7	2	1	0.924937	1.046508	0.942946	0.901753	0.950044	0.961342	0.943207
Q1HVA2;E1ZT20;D8U9J4;A8HP84;Q1HVA0;B1PL92;I0YMA8;B0JHH3;K8E991;A4RQR7;Q20FC5	1	1	1.093909	2.296513	2.295813	0.944319	1.83352	0.873616	0.901266
Q1KVV6;E9NPV5	1	1	1.020887	1.679082	1.789599	0.96335	1.841467	2.070149	2.251781
Q1KVY3	1	1	0.859509	1.779202	1.898729	1.905772	2.473662	1.849002	2.426731
Q84X75;E1ZFR4;D8TK78	1	1	1.036619	1.105898	1.030114	0.924936	1.025198	1.056242	1.031883
A4RZD2;I0Z4C1;	1	1	1.17996	0.993526	0.927829	0.929237	0.90852	0.941834	1.031464

K8EEU7;E1ZFG9									
A8HW56;D8TIS4;C1MLD8;I0YZZ5	1	1	0.892984	0.991846	1.10792	1.224594	1.200162	1.084064	1.065284
A8HZZ1;D8TM26	1	1	1.127936	1.078804	1.056189	0.927859	1.038246	1.123835	1.08337
A8I972	2	1	0.989861	1.026305	1.004805	1.213912	1.205172	1.046713	1.194997
D7FWI4;I0YNR0;C1MVY5;K8EN77;A4RSQ1;D8UC14;A8HRZ9;E1Z345	1	1	1.021541	1.142956	1.254656	1.13678	1.212133	1.084515	0.974157
D8TLB0;A8J1U1;Q002K0;Q002J6;Q002K1;Q002J7;Q002K2;Q002J8;Q002K3;Q002J9;A7M6Q3;D8LHY7;A8I7T1;D8TT40;Q66T67	1	1	0.874137	0.798598	0.869468	1.199509	0.872029	0.846711	0.962844
D8TLH8	1	1	0.915461	0.995832	1.085627	0.936313	1.131313	0.813311	0.887556
D8TM08	1	1	1.057508	1.174123	1.129819	0.74952	1.008399	0.981251	0.899571
D8TZZ8;A8JIB7;E1ZRV3	2	1	1.165873	1.359866	1.375482	1.13156	1.522944	1.345589	1.26721
D8UBQ8;Q9LLL6	1	1	1.094688	0.65613	0.733384	0.697906	0.688483	0.925292	0.802442
D8UDE0;I0Z891	1	1	1.046942	0.760194	0.711629	0.816331	0.669801	0.80218	0.81598
D8UI88	1	1	0.968512	1.074692	0.998037	1.267985	1.282196	1.240413	1.375211

E1Z349	1	1	1.024777	0.945616	0.954206	1.217144	1.027112	1.044326	0.928085
E1ZQ02;I0Z789	1	1	1.009105	1.152491	1.124208	1.004428	1.565776	0.995356	0.975597
G4WUV9	1	1	0.99936	0.980102	1.052006	1.043645	1.078499	0.784185	0.821154
I0YIF2	1	1	0.974333	0.950659	0.84191	0.94897	0.728995	0.808459	0.800829
I0YIX7	1	1	0.886022	1.002308	1.177169	0.947065	0.917134	0.751688	0.945513
I0YX80;D8UIY5	1	1	1.104997	1.247467	1.156135	0.87772	0.836918	0.904664	0.872832
Q1KVT0;P06541;D0FXY0;A0A1C8XRG2;Q8HDD9;K8FHJ4;F2YGR0;E9NPS5;B0JFM7;Q8HDG4	1	1	0.873497	0.544774	0.588623	1.076564	0.682091	0.819859	0.735254
Q75VY8;D8UAY7	1	1	0.879644	0.626894	0.692502	0.891902	0.707015	0.802091	0.766701
Q9FNS5;D8U926;E1Z366;C1MSW4	1	1	1.07713	1.12541	0.78625	0.923806	0.924194	0.947424	0.956097
S4VNM6;H6X2F8;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;W6AAY4;W6AAZ3;A0A0X9AMW9;A0A0X9AGK8;E9NPX3;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;W6A299;Q1KVV0;F2YGL1;A0A1	1	1	1.08958	0.980354	1.01103	0.344385	0.984198	0.941314	0.94947

72C918;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;P00877;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1VNR7;M1VK51

;M1VK44;M1VEJ4;M1VEI8;Q3S3F0;Q2I3J7;Q8HD99;W6A241									
A4RTP0;C1MJJ1;K8FCT0;E1Z349;I0Z036	1	1	1.010869	1.077709	1.064685	1.138195	1.089483	1.063386	1.231233
A8I4P5;A4RVP7;C1MQ23;K8F6X3;D8TIE9	1	1	0.825921	1.181365	1.081474	1.128719	1.242015	0.928709	1.11814
A8IQU3;D8TRA2;E1ZS63	1	1	0.696585	0.525865	0.690654	1.181606	0.753445	0.925431	1.127963
A8J1T4	1	1	1.020916	1.140569	1.200994	1.025352	1.187247	1.166007	1.050292
D8TK12;A8IE23;B6E5W6	1	1	1.129459	0.933327	0.91306	0.947654	0.669704	1.186196	1.083557
D8TK12;A8IE23;E1Z520;D8LQV8;A4S521;K8ENB0;B6E5W6;I0Z5K3	1	1	0.991634	1.126815	0.982894	1.005562	0.908377	1.090265	1.137626
D8U224;A8IHX1	1	1	1.03519	0.943281	0.956811	0.979526	1.03777	1.157133	1.095357
E1Z824	1	1	1.097334	1.030092	1.099526	1.184551	1.183727	1.006567	0.979518
P02769;CON__P02769;CON__P02768-1	2	1	1.01079	1.177982	1.296877	1.221696	1.011637	1.012573	1.218319
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3;A0A1C8XRL7	1	1	1.16256	1.179629	1.393499	1.494607	1.6272	1.287468	1.551728

Q8RY44	1	1	1.10273	1.270224	1.101994	0.61208	1.121203	1.036502	1.140058

iTRAQ#2

	# uniq pepts	Q (lin, MC)							
	22	113	114	115	116	117	118	119	121
A0A097PB89;D1J797;B0JWV1;P48080;E9NPZ5;D8LJM3;P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3;F2YGQ9;Q1KVU0	1	1	0.973601	1.065346	1.042408	1.019655	0.893232	0.948719	0.911439
A0A0C4K0H7;I0YIH9	2	1	0.876554	1.084211	1.123576	1.210257	1.02541	1.097353	1.102328
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;M1VNR7;M1VK51;M1VK44;M1VEJ4;Q3S3F0;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;F2YGL1;A0A172C918;P00877;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V73	1	1	0.953172	1.18542	1.142547	1.247704	0.98703	0.832901	0.764752

9;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1V8T0;M1VEI8;Q2I3J7;Q8HD99;W6A241									
A0A0X9AMW9;	2	1	0.834927	0.577737	0.83438	1.576106	1.377884	0.649377	0.628839

A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;A0A172C918									
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;F2YGL1;Q8HD99;W6A241	4	1	1.090975	0.897581	1.032427	0.995607	0.907131	0.769712	0.739919
A0A172C1L3;Q1KVS9;A0A120N1C6;A0A172BZR9;A0A110B8L5;A0A0X8XG29;A0A110B817;A0A120N1C5;P17245;A0A097PBA2	1	1	1.193539	1.003165	1.061568	0.390216	0.313212	0.819741	0.776607
A4RQQ6	1	1	0.939433	0.815344	0.90754	1.525541	1.679514	0.863054	0.983798
A4RTP0;C1MJJ1;K8FCT0;E1Z349;I0Z036	1	1	0.976091	0.95337	1.099972	0.797225	0.93395	0.883407	0.935727

A4S5T5	1	1	0.972809	1.03793	1.029093	0.809538	0.903119	1.037063	1.003931
A4S9U1;K8F313	1	1	1.128119	1.049635	0.912749	0.802876	0.828831	1.25746	1.169441
A8HW56;I0YZZ5	1	1	1.005337	0.840816	0.694879	0.920634	1.34286	1.494729	1.099568
A8HXL8;E1ZEB1;D8TI16;I0ZA63	1	1	1.078354	0.854193	0.662183	0.863017	1.034308	0.97343	0.867694
A8HY43;D8THL7	1	1	0.714494	1.026452	0.903103	0.945736	0.985469	1.375404	1.389118
A8HYU5;C1N037	1	1	1.002886	0.986306	0.99228	0.802723	0.921232	1.149049	1.384242
A8IHL3;D8U3T1	1	1	0.986708	1.000918	0.963941	0.878003	0.874134	0.974312	0.928748
A8IQU3;D8TRA2;D7FXG1	1	1	0.988832	1.299614	1.102184	1.291178	1.413885	1.304663	1.32041
A8IQU3;D8TRA2;E1ZS63	1	1	1.111599	1.101254	1.135419	1.15456	1.101346	1.048163	1.229469
A8IRT2;I0YSF0;C1MLJ8;E1ZTE2;D8TV91	1	1	0.872037	0.530104	0.799363	0.913562	1.064866	0.822532	0.863619
A8ISB0;A8ISA9;D8TSY0;D8TK58;I0Z3J7	1	1	1.047919	1.072729	1.1263	1.214008	1.341563	1.286313	1.249419
A8IX80	1	1	1.047548	1.429414	0.85167	0.905004	0.867124	0.758039	0.896044
A8IX80;D8UGB5	2	1	0.951176	0.855539	0.930394	1.070771	0.914784	0.898269	0.96642
A8IXE0;D9IUM4	1	1	1.042049	1.081063	1.191829	1.133295	1.022182	1.01162	1.032284
A8IZU0;D8TMR1	2	1	1.293762	1	0.968937	1.647512	1.105231	1.170046	0.918498

A8J6C7;D8TTK4;I0Z5Q8	1	1	0.98273	0.996563	0.927164	0.878682	0.890447	0.951231	1.009532
A8JDV2;D8UIE7	2	1	0.906171	1.222603	1.09257	1.109331	1.166615	1.096388	1.112091
A8JEU4;Q8RY44	2	1	1.221257	0.566483	0.776332	0.8142	0.821424	0.872665	0.822466
A8JHX9	2	1	1.0167	0.994524	1.013582	0.896955	0.887251	0.792018	0.737671
B7TJI2	1	1	1.034627	1.050191	0.808035	1.166631	0.91622	0.836421	0.805114
C1MNA2;D8U0E5	1	1	1.352191	1.232169	1.148056	0.176568	0.126955	1.413006	1.38169
C1MVX0	1	1	1.039748	1.144866	1.260954	1.092964	1.05267	1.028914	1.084277
C1N5S1	1	1	1.090741	1.201057	1.14248	0.948209	1.164503	1.271787	1.225239
C1N789	2	1	0.829959	1.637427	1.560693	1.770293	1.563792	1.283388	1.449876
CON__P00761	4	1	1	1.127349	1.071943	1.071938	1.088295	1.036173	1.032186
CON__P04264	2	1	0.925778	0.862492	0.923038	0.953045	1.004876	1.272672	1.112277
D7FK90;D8LI58;D7FZN2;I0YNC4;I0YKI7;P93662	1	1	0.961424	1.001798	0.938321	0.776697	0.771055	0.917877	0.840521
D8TJ31	1	1	0.985251	1.184134	1.149246	1.174865	0.968591	0.912201	0.878052
D8TN65;A8IJ19	1	1	1.02123	1.112989	1.124223	1.190617	1.063921	1.141717	1.053698
D8TV46;A8IRQ1	1	1	1.014614	0.974655	0.9917	0.926771	0.930582	1.113027	1.039204
D8U1F3;A8IW39	2	1	1.118714	0.950554	0.949622	0.771074	0.950761	1.081675	1.059174
D8U1I3;I0YVA0	1	1	0.942063	1.070921	1.003116	1.139329	0.94522	0.990341	0.979657

D8U477;D7FRY5;A8ILN4;A4S2B3;C1MNJ9;K8EKA1;I0Z4W2	1	1	0.930189	0.838335	0.790817	0.450329	0.895276	0.883607	0.83586
D8U477;D7FRY5;A8ILN4;I0Z4W2	1	1	0.824601	1.076277	1.028448	1.082276	1.170326	1.039266	1.021777
D8U4Q1	2	1	0.906702	1.044161	1.014623	0.789636	0.931821	1.135367	1.145198
D8U5B1;A8JG03	6	1	1.252685	0.792415	0.740498	0.865596	0.683915	0.809073	0.840036
D8UC42;A8IA45;I0Z9U5	3	1	1.056388	0.9352	0.941143	1.00348	0.996149	0.954022	0.967567
D8UI03;A8HYV3	1	1	1.047385	1.165027	1.053074	0.979012	0.917501	0.976966	1.032323
D8UI03;A8HYV3;E1ZE03;Q8VY41;Q9M452;I0Z190	1	1	0.800878	0.916689	0.83447	1.789496	1.505734	0.859865	0.878388
E1Z746	1	1	0.921332	0.785656	0.851342	0.738115	0.988787	0.936926	0.952855
E1Z824;I0YWB9	1	1	0.866786	0.852574	0.908177	1.228977	1.104916	0.910411	0.791366
E1ZBK2	1	1	0.916221	1.013373	0.795184	1.101124	0.994497	0.869048	0.821395
E1ZBK2;D8TNN3;D8THW4;A8HX38	3	1	1.03585	0.703238	0.798563	1.143358	0.981676	0.958769	0.969647
E1ZBK2;D8TNN3;D8THW4;A8HX38;K8F4B8;A4S6B6	1	1	1.092916	0.657408	0.695179	1.184551	1.082026	0.8979	0.938113
E1ZBK2;D8TNN	1	1	0.738621	0.475168	0.966395	1.812657	2.172058	0.775878	0.831507

3;D8THW4;A8HX38;K8F4B8;A4S6B6;C1MZI5;C1MT59									
E1ZJQ8	1	1	1.029575	0.775004	0.797158	1.079016	0.954706	0.953881	0.905501
E1ZQL8	1	1	0.973174	0.981899	0.873251	0.801101	1.164758	1.216698	1.327753
E1ZSU0	1	1	1.012616	1.145251	1.306461	2.127117	1.4541	0.97811	1.011041
I0YP36	1	1	1.069721	1.102731	1.057757	0.943223	0.821671	1.040952	0.894248
I0YPF7;E1ZM95	1	1	1.163297	0.835461	0.629398	0.535917	0.478518	0.957683	0.974795
I0YQ64;A8J537	1	1	1.079019	0.96667	0.939639	1.516995	1.279734	0.944133	1.019573
I0YRY7;Q56D00;E1ZIV3	1	1	1.079751	1.427467	1.2466	1.062837	0.977186	0.9425	0.967987
I0YS06;H2ELS9;D8TSK8;A8JHQ7;C1MIT8	1	1	1.114978	0.491029	0.65622	1.577832	1.917361	0.737802	0.842872
I0YV40	1	1	1.115193	1.309559	1.355324	1.176079	1.340613	1.458355	1.518566
I0Z1U0	1	1	1.039351	1.081741	1.185586	1.20793	0.995998	1.179083	1.07276
I0Z401	2	1	0.884412	0.771664	1.023791	1.195665	1.066235	0.867174	0.829825
I0Z6P1;A8HYD2	1	1	0.91139	0.877302	1.033018	0.503103	0.914546	1	0.985384
I0Z918	1	1	1.077002	0.940222	0.995644	1.206475	1.208305	1.105589	1.161024
K8EQX0;A4RVI7;C1N9S9;I0Z698	1	1	1.179322	1.192198	1.055999	0.925084	0.901283	1.258029	1.118323
K8FA09;C1MHY2	1	1	0.856491	0.989373	1.032973	0.988829	1.136259	1.357133	1.103584

P02769;CON__P02769	22	1	0.982862	1.046705	1.403838	0.807614	0.970631	1.014074	0.98224
P06007;Q1KVW6	1	1	0.959795	0.944994	0.885173	0.968302	0.991536	1.123759	1.215056
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3;B2LWG0;D8UK13;Q8SLI8;Q1KVU0	1	1	1.006003	1.538817	1.254669	0.966194	1.049176	1.262437	1.293382
Q1KVT0	2	1	0.931491	0.921839	0.915668	1.342042	1.026069	1.068095	1.05529
Q1KVT0;P06541;K7NVH0;D0FXY0;A0A1C8XRG2;Q8HDD9;Q8HDG4	3	1	0.920933	1.204061	1.17747	1.062844	1.06558	1.069394	1.025679
Q1KVU8;F2YGK0	1	1	0.778833	0.890774	1.074138	0.888966	0.980906	1.041406	1.089932
Q1KVV6	1	1	0.751566	1.353465	1.453718	0.93193	1.631098	1.767019	2.121381
Q1KVY1	1	1	0.92227	1.346012	1.643765	1.036928	2.152082	3.271237	3.774438
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3;A0A1C8XRL7	1	1	1.122954	1.237099	1.216301	1.004316	1.028324	1.134948	1.066594
Q42690	1	1	0.915459	1.150472	1.386864	0.97235	1.210567	1.274921	1.51443
Q42690;D8TKY4;I0YN66;E1ZQQ5	2	1	1.323095	0.715357	0.616242	0.976834	0.803381	0.855298	0.784814

Q84RL9	2	1	0.940117	1.242772	1.202592	1.291621	1.039108	0.929971	0.90466
Q8HDG4	1	1	0.986598	1.181902	1.222254	1.221995	1.086025	0.97727	0.951636
Q8RYB9	1	1	0.833729	1.437768	1.436335	1.077837	1.085785	1.03378	1.070924
Q96550;D8UD45	2	1	0.844759	0.963222	0.833029	0.902097	0.830555	0.762925	0.808297
S4ULQ5	1	1	0.953243	0.974788	0.977731	1.225636	1.061769	1.027046	1.032031
S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;F2YGL1;A0A172C918;P00877;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I	1	1	1.173598	0.901119	0.905809	0.831239	0.720468	0.595022	0.602026

3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1V8T0;M1UZB8;M1VEI8;W6A1S2;P24312;A0A023SZZ9;A0A0A0Y7C9;A0A1S6M237;A0A140CQM1;R4ITL5;A0A140CQM0;Q2I3J7;Q8HD99;W6A241									
A0A097PB89	1	1	1.077186	1.056631	1.129057	0.995982	0.824774	0.816893	0.734051
A4S734	1	1	0.840345	0.787906	0.948952	0.906301	1.130998	1.189141	1.194333
A4S7X2;K8EHR6;C1N6J0	1	1	0.954741	0.916576	0.912192	0.948204	1.053269	0.906454	0.951389
A4S824;D8UF17;A8IWK2;K8F1R7;C1MYV2	1	1	1.044154	1.512154	1.490947	1.099089	1.561003	2.30865	2.802527

A8HZZ1;D8TM26;E1ZCK4	1	1	0.919368	0.743085	0.82268	1	1.031586	0.912188	0.867438
A8IQU3;D8TRA2	5	1	1.003778	0.985132	0.971132	0.959884	0.819848	0.880857	0.852695
A8IQU3;D8TRA2;E1ZS63;I0YLJ1	1	1	0.91805	0.857502	0.961075	1.00872	1.041937	0.878182	0.977242
A8IWQ7;D8UEY8	1	1	0.971612	0.490124	0.658376	0.704373	0.724613	0.759785	0.829238
A8IYP4;D8TRR7	1	1	0.985884	1.066136	0.993511	1.020738	0.87438	0.891283	0.801664
A8J6K9	1	1	1.802499	0.649835	0.721522	1.27486	1.602295	0.806715	0.716179
A8JDN2;E1ZQS3	1	1	1.08469	0.885276	0.930002	0.908334	0.994921	0.917739	0.924616
C1MHD4;E1ZGF5	1	1	0.968367	0.99401	1.188724	1.130027	1.128993	0.897807	1.181992
C1MU18;A4RYP4	1	1	0.959487	0.794655	0.866927	0.754627	0.710923	1.078411	0.915291
C1MYV3;E1ZLQ3;I0YI95;D8UA08;A8JAV1;Q9SWF3;O003989;D7FQK6	1	1	0.964125	0.857379	0.886178	0.818193	0.882751	1.02563	0.986742
CON__P13717	2	1	0.89867	1.233789	1.205913	0.842314	1.022747	1.206639	1.140053
D8TQM8;A8J3Y6	1	1	1.014477	1.198596	1.063258	1.48917	1.699086	1.382971	1.32912
D8TTA3	4	1	1.001678	1.12265	0.971008	0.9435	0.844221	0.944596	0.900419
D8TTX1	1	1	1.038162	0.955744	0.956215	0.958876	0.994049	1.067208	1.014954

D8TV46;A8IRQ1;E1Z7C4	2	1	0.830805	0.629425	0.684293	1.263731	1.333096	0.886654	0.768242
D8TW10;E1ZKW6	2	1	1.037986	0.930884	0.98853	0.961974	0.880803	0.945606	0.920972
D8TZU3;Q6SA05;E1ZSL5	1	1	1.116846	1.058506	0.809694	0.930283	0.926448	1.016251	0.977031
D8U1R3;E1ZP98;I0Z1A5;C1N726;A4SAW5;K8F2G0	1	1	1.230426	1.003456	0.994742	0.892378	1.065766	1.014649	1.076234
D8U973;A8IZW6	1	1	1.075742	1.117416	1.174044	1.235267	1.241459	1.142528	1.275711
D8UDE0;A8HPL8;E1ZJ54	1	1	0.987846	1.070674	1.105232	1.081452	0.988911	1.055667	0.968484
D8UFR3;A8J9T0	3	1	0.917042	1.033607	1.017828	0.896708	0.939465	0.93444	1.015773
E1Z356	1	1	1.125316	0.59574	0.724339	1.416326	0.983424	0.862557	0.843571
E1Z5I7;A8IZZ4;D8U995;E1Z8A6;D8U547;A8JF18_CHLREUbiquitin,minorisoformOS=Chlamydomonasreinhardtii GN=UBQ1a;E1ZHZ0;A8JCX9;D8UEE9;A8JF17_CHLREBi-ubiquitin,majorisoformOS=Chlamydomonasreinhardtii GN=UBQ	1	1	1.040654	1.120568	1.108965	1.149216	1.090054	1.069064	1.082787

1a;I0YMQ7;D8U474;I0Z619;E1ZCE0									
E1Z6L2	2	1	1.030001	0.975046	1.031719	1.239464	1.48704	1.149038	1.153267
E1Z7C4	1	1	0.994994	0.96134	0.897349	0.894302	0.85513	0.946642	0.942591
E1ZFQ1	1	1	1.03293	0.871666	0.841257	0.723223	0.800062	0.934698	1.01931
E1ZMW8	1	1	0.954181	0.600747	0.797871	0.725079	0.954758	0.874972	0.832587
I0YL77;E1ZL24	1	1	0.988744	1.038174	1.158059	1.296119	1.249564	1.088877	1.011741
I0YL77;E1ZL24;D8TPC8;A8IL29;A4S5Z2;C1MH11;K8FE75	1	1	1.054004	0.948078	1.043989	0.950423	1.172312	1.138115	1.023427
I0YNY7	1	1	0.996085	1.3349	1.434816	0.79031	0.739392	1.531422	1.598336
I0YWB9;Q6J213	1	1	1.033603	0.825613	0.663309	1.188179	0.960296	0.780826	0.744024
I0YZ27	1	1	1.057695	1.087536	0.972534	1.173866	1.135213	1.093447	1.102889
I0Z3A2	1	1	1.00547	1.28345	1.351135	1.226229	1.106398	1.086631	1.029348
K8F4N5	1	1	1.021744	0.742376	0.935946	1.03695	1.030806	0.898021	1.030957
P06007;Q1KVW6;Q4JLT1;K8FE34;K7NRG3;F2YGQ0;E9NPS3;D0FXW8;A0A1C8XRK9;D1J6Z4;B0JR69;P48079;A0A097PB60	1	1	1.040653	0.764738	0.847701	0.946338	1.019436	0.960933	1.05746
Q00914;K7NRF9	2	1	1.081668	1.937587	1.759677	1.236994	1.655633	2.171136	2.248796

;D1J7C7;D0FXW7									
Q1KVT0;P06541;K7NVH0;D0FXY0;A0A1C8XRG2;Q8HDD9;K8FHJ4;Q8HDG4	1	1	1.249642	1.162251	1.004158	0.939548	1.097745	2.043549	2.236245
Q1KVT0;P06541;Q8HDG4	3	1	0.861679	0.977108	1.071714	0.996413	1	0.872622	0.889935
Q1KVT0;Q8HDG4	1	1	1.06303	1.359992	1.18666	0.565726	0.505026	1.241281	1.331664
Q1KVY2	2	1	0.830874	0.707647	0.750252	1.100669	1.745349	0.98269	1.175939
Q763T6	1	1	0.907044	1.92241	1.938453	1.04727	1.502322	2.619374	2.617734
Q8HDD7	1	1	0.984859	0.796884	0.856462	0.678616	0.990533	0.909387	0.963242
Q9FE86	2	1	0.920682	1.354868	1.082612	0.99754	0.94578	1.310354	1.299214
S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;F2YGL1;A0A172C918;P00877;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M	1	1	0.958991	0.94456	0.986803	1.259221	1.169716	0.805787	0.833525

1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1V8T0;M1UZB8;M1VEI8;W6A1S2;P24312;A0A023SZZ9;A0A0A0Y7C9;A0A1S6M237;A0A140CQM1;R4ITL5;A0A140CQM0;S4VV39;Q2I3J7;Q8HD99;W6A241	

A0A0S1LH61	1	1	1.053117	0.711504	0.733554	1.420012	1.47169	0.729136	0.722015
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;M1VNR7;M1VK51;M1VK44;M1VEJ4;Q3S3F0;Q31912;A0A023T0H1;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;P00877;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V737;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M	1	1	0.993077	1.295557	1.281377	1.105076	1.155872	0.983785	1.022752

3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;K7NSN7;D0FXZ7;A0A1C8XRQ3;M1V8T0;M1UZB8;W6A1S2;P24312;A0A023SZZ9;Q8HD99;W6A241;A0A023SYL4									
A0A0X9AMW9;A0A0X9AGK8;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;A0A172C918	1	1	1.042009	0.768013	0.794177	0.928778	0.74609	0.527845	0.560454
A0A172C1L3;Q1KVS9;A0A172BZR9;A0A0X8XG29;K7NSQ0;D0FXV6;A0A1C8XRX1;A0A120N1C5	1	1	1.550358	2.247982	1.528395	0.604472	0.549199	1.042707	1.037062
A4RQU1;C1MGL	1	1	0.97709	1.100997	1.024218	0.835375	1.014674	1.081632	1.086851

0;K8E9P2									
A4RTP0	1	1	1.002518	1.007214	0.89724	0.946017	0.853314	0.877246	0.891508
A4RTP0;C1MJJ1;D8U848;A8ICG9;E1Z349;I0Z036	1	1	1.049139	1.316273	1.327659	1.289549	1.374832	1.067729	1.057509
A4S0V1	1	1	1.02491	1.116407	1.009535	1.011109	0.863052	0.919614	0.950882
A4S614;C1MJ74	1	1	0.98289	1.39543	1.321432	1.314966	1.140571	0.917054	1.053639
A8HZZ1;D8TM26	1	1	0.941428	0.919542	0.817326	0.705364	0.81643	1.007093	0.961947
A8IQU3;D8TRA2;K8EN95;A4RSS5;C1MKD5	2	1	1.010122	1.016731	1.004352	0.96492	0.927992	0.943158	0.929742
A8IQU3;D8TRA2;K8EN95;A4RSS5;C1MKD5;I0YLJ1	1	1	1.020612	1.026116	0.991918	1.098223	1.184643	0.915562	0.931755
A8IRT2	1	1	1.053151	0.602471	0.662404	0.788166	0.695819	0.912251	0.980236
A8IZU0;D8TMR1;A4RWG3;K8F7J7;C1MP69	1	1	1.10433	1.097307	1.12939	0.960752	0.994745	1.121872	1.069499
A8J146;D8UAK0	1	1	1.115056	1.277171	1.18152	1.178435	1.021205	1.113992	1.038974
A8J1M9;D8TL63	1	1	1.027592	1.174796	1.033825	0.904466	0.945877	1.258849	1.189217
A8J237	1	1	1.013142	1.153649	1.067629	1.127101	0.961787	0.937932	0.836483
A8J6C7	1	1	1.149866	1.235862	1.603547	1.167875	1.283985	1.370153	1.423875

A8J6K9;D8THE2	1	1	1.063542	1.328792	1.258876	1.490983	1.442541	1.066047	0.97191
C1MHU2;K8ENS6	1	1	1.214656	0.951199	0.804805	0.612307	0.830567	0.893895	0.934962
D7FUD3	1	1	1.076545	0.60782	0.721735	0.54066	0.713454	0.737087	0.993204
D8TK77	1	1	0.874942	1.263183	1.27244	1.043698	1.371837	2.181591	2.184608
D8TPD5;A8IL08;K8EJA2;I0Z5A8;E1ZSS5;A4S2T2;C1MNR3	1	1	1.04039	1.253248	1.175073	1.124709	0.958684	1.148395	1.009361
D8TWH5;A8JFB1	1	1	0.748141	0.75422	0.756252	0.72039	1.477729	0.964515	0.973982
D8TWH5;E1ZRQ7	2	1	0.955846	0.929367	1.08745	0.915434	0.996813	0.970893	1.099248
D8TZD7;A8ITH8;I0YWY2;K8EB57;E1ZRQ6	1	1	0.883298	0.919652	1.142821	1.022379	1.246001	0.97568	0.905822
D8U477;D7FRY5;I0Z4W2	1	1	1.04101	0.968894	1.27122	0.753967	1.041893	1.14247	0.949124
D8UBQ8;Q9LLL6;E1Z2N8;I0Z0D7;K8EU58	1	1	0.878784	1.060014	1.088423	1.332467	1.187815	1.09722	1.077119
D8UF03	1	1	0.951533	0.746566	0.61287	0.7492	0.695958	0.725743	0.736489
D8UFZ3;E1Z4A2;A8J9S7	1	1	0.992409	0.971755	0.999909	0.852759	0.91621	1.078959	1.01742
D8UI03;A8HYV3;E1ZE03	1	1	1.122144	0.820138	0.750542	0.912357	0.881884	1.025959	0.855961

E1Z4F7	1	1	0.895069	0.878104	0.92475	1.343769	1.51735	0.863021	0.901144
E9NPW9	1	1	0.94062	0.950871	1.025406	1.039693	1.093655	1.075934	1.02959
I0YR21;E1ZPY6	2	1	0.926343	1.273	1.252804	1.226234	1.075149	0.953597	0.957728
I0YZE5	1	1	0.970282	1.323907	1.28157	0.77169	0.913429	1.086251	1.088959
I0Z0B3	1	1	1.015794	0.851398	0.960214	1.132524	1.327882	0.95473	1.009088
I0Z4W2	1	1	0.96512	1.165948	0.996839	1.090314	0.924765	0.96247	1.060229
I0Z5X3	1	1	0.911	0.956436	0.709531	0.586835	1.285843	1.492269	1.367225
K8EM49;E1Z926;A4S6Z0;C1N1J6	1	1	1.019279	0.907126	1.151129	1.227935	1.46216	1.581404	1.653267
K8F0N5	1	1	0.917075	1.001028	0.931201	0.697412	0.769611	1.150758	1.195306
P48101;A0A097PB99	1	1	0.980092	0.816003	0.873194	0.753224	0.833319	0.970453	0.920573
Q1KVS9;P17746;K7NSQ0;D0FXV6;A0A1C8XRX1	2	1	1.096833	0.80632	0.749425	0.829069	0.869333	1.274072	1.207121
Q1KVT0;P06541;K7NVH0;D0FXY0;A0A1C8XRG2;P48081;A0A097PBH6;F2YGR0;D1J7B4;E9NPS5;Q8HDG4	1	1	0.953589	1.097199	1.144753	0.901047	0.967015	0.975723	1.062048
Q1KVT0;P06541;P48081;A0A097PBH6;Q8HDG4	1	1	0.912467	0.338598	0.436583	0.433453	0.606736	0.569913	0.621731

Q1KVU3	2	1	0.8288	0.66985	0.647982	0.591056	0.791472	0.932594	1.037788
Q1KVX3;K7NSN1;A0A1C8XRP4	2	1	0.884387	0.869865	0.915989	1.062482	1.468456	1.070235	1.173452
Q6J213	1	1	0.808028	1.067491	1.229605	1.036452	1.374611	1.721089	1.74035
Q763T6;E1ZRI5;D8U7C0;I0YKU6	1	1	1.00318	0.87471	0.941585	0.918433	0.860213	0.970086	1.016992
Q8LRU1;I0YP34;D8TX08	1	1	0.938578	0.988549	0.922046	0.927094	1.105233	0.975591	1.047857
Q9FEK6	1	1	1.060316	0.779519	1.055189	2.310676	2.374023	1.06543	1.069059
A8HNE8;D8UHM8	1	1	1.122827	1.456252	1.591736	1.338156	1.387514	2.357128	2.437463
A8J5P7;D8TNA2	1	1	0.811253	0.748446	0.830988	0.916064	0.95023	1.065623	0.943852
A8J7F6;D8TUP1	1	1	1.076475	1.059581	1.019625	0.938395	1.02667	1.135418	1.197818
A8JCY4;D8U593;I0YSE8	2	1	0.929823	0.840165	0.831299	0.904019	0.846477	0.830001	0.711362
A8JEU4	2	1	0.919555	1.135849	1.139741	1.025749	0.939451	0.729161	0.76094
D4N535	1	1	1.121202	0.523535	0.518938	0.596802	0.510045	0.540966	0.680939
D8TP83;I0YQQ4;A8IKP1;E1ZQ26	1	1	1.02019	0.987387	0.994165	1.341404	1.021279	1.012953	0.974104
D8TVP4	1	1	1.194472	0.607948	1.1511	1.471765	1.351114	1.722822	1.38267
D8TZU3;A4RW20;E1Z378;K8F6A2	1	1	1.195309	0.744587	0.957185	0.885847	0.981608	0.879409	0.972131

D8U4B4;A8J597	1	1	1.016456	0.740326	0.835951	1.034634	1.066113	0.845259	0.844557
D8U4Q1;E1ZGR1;A8IAN1;K8ERB6;I0YJZ4	1	1	0.916703	0.831785	0.869036	0.588535	0.995354	0.971458	0.928513
D8UE23	1	1	1.271426	0.187481	0.396133	1.06133	0.99075	0.569663	0.629749
D8UI03;A8HYV3;E1ZE03;Q8VY41;Q9M452	1	1	0.966287	1.057989	1.006224	1.009154	0.992831	0.905655	1.025317
E1ZNM7;I0YMX2;A8J8B3;D7G034;K8F1Y0	1	1	1.051653	1.157505	1.087163	1.005361	1.077601	0.973382	1.023151
E1ZQL8;D8TUG4;K8EP91	1	1	1.040406	0.965534	0.917383	1.097398	0.981059	0.859109	0.856891
E1ZRA9	1	1	1.020978	0.788808	1.006383	1.201285	1.074339	0.843213	0.965823
E1ZT16	1	1	0.932183	1.095457	0.967157	1.10644	0.909961	1.026123	0.807711
I0YKI7;A8JEU4;Q8RY44	1	1	1.01936	1.053137	0.950334	0.999836	1.119932	0.992811	1.072645
I0YRY7;Q56D00	1	1	1.171588	1.509558	1.322143	1.256636	1.480529	1.139603	1.035881
I0YSP0	1	1	1.038924	1.145766	1.007218	1.17344	0.938099	0.929986	0.870393
I0YTX9	1	1	0.974577	0.644209	0.69385	1.095884	1.096212	0.721869	0.749411
I0YUW3	1	1	0.880736	0.918469	0.779115	1.271078	1.234753	0.932107	0.842874
I0YX80	1	1	1.082354	0.800095	0.855771	1.133125	1.044975	1.053903	0.959764
I0YZE5;C1ML90;A4RRH9;A8IDP6	1	1	0.877362	0.886374	0.969569	0.934593	0.827059	0.856346	0.869113

;Q39708;D8TKN5;K8ENP9									
I0YZZ5	1	1	0.971773	0.735638	1.028186	0.88569	1.017612	1.019361	1
I0Z028;D8U1H9;E1Z342;A4RTQ1;K8EP00;D7FP46	2	1	1.052927	0.718563	0.775918	0.811135	0.7311	1.049781	0.941546
I0Z4M6	1	1	0.885893	1.091769	1.146027	1.433229	1.106288	1.358091	0.990981
I0Z5T7;E1ZN46	1	1	0.934611	0.981451	0.861727	0.861724	0.903562	1.016973	0.973783
I0Z849	2	1	0.84319	0.98837	1.053315	1.161492	1.146491	1.044681	0.96418
K4EKL3	1	1	0.929371	1.001793	0.880001	1.29452	1.069555	1.067815	0.930131
K8ENF9	1	1	1.110602	0.884365	1.145082	1.137859	1.252895	1.103817	1.151501
P26526;B7U1J0	2	1	1.025392	0.987852	1	0.812504	0.91915	1.042081	1.173067
P37255	1	1	0.635293	1.001828	1.093075	0.938217	1.271824	1.065957	1.012644
Q1HVA2;E1ZT20;D8U9J4;A8HP84;Q1HVA0;B1PL92;I0YMA8;Q8VXQ9	1	1	0.888172	1.122145	1.713002	1.066922	2.198643	2.316683	2.531262
Q1KVS9	2	1	0.788922	1.039286	1.4041	0.830084	1.108218	1.32721	1.217747
Q1KVT0;P06541;K7NVH0;D0FXY0;A0A1C8XRG2;Q8HDD9;P48081;A0A097PBH6;K8FHJ4;F2YGR0;D1J7B4;E9NPS5;	1	1	1.04671	1.137843	0.991926	0.718249	0.863204	1.11111	1.059991

Q8HDG4									
Q1KVT2;E9NPX5;D1J798	1	1	0.959949	0.981742	1.0253	0.843013	0.954256	1.069048	1.008075
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3;A0A1C8XRL7;W8E1S1;B0JR68;F2YGQ1	1	1	0.822353	1.026805	0.971264	0.947042	1.976129	1.706695	1.779922
Q9XGU3;D8U5D0;G4WUW0	1	1	0.935334	1.474078	1.46548	1.006632	1.456478	1.966505	2.216331
A0A1B0VE51	1	1	0.976356	0.973496	0.982237	1.167563	1.364591	0.913077	1.015298
A4SB22	2	1	1.042201	1.18159	1.241204	0.948299	0.986008	1.070403	1.001765
A8HS14;E1ZTI5;D8TZQ2;I0Z1V7	1	1	1.149735	1.114015	0.981253	0.930065	0.781399	0.942531	0.975285
A8IN95;D8TLU2	2	1	0.852659	0.669937	0.859467	0.642495	0.72078	0.828115	0.79866
A8IZU0	1	1	1.080969	1.235085	0.975305	0.764887	0.904512	0.985352	1.066694
A8IZU0;D8TMR1;B7TJI1;D8UI03;A8HYV3;E1ZE03;Q8VY41;Q9M452;I0Z190;C1MVP3;K8ENF9	1	1	0.9949	1.150703	0.915488	1.167168	1.34217	1.025143	1.226934
A8JDW2;D8U3S7	1	1	0.972444	1.314054	1.263516	1.110803	1.134053	1.087214	1.00033
A8JFZ0_CHLRESerineglyoxylate aminotransfera	1	1	1.031071	1.028879	0.846188	1.043673	0.871039	0.870112	0.788354

seOS=Chlamydomonasreinhardtii GN=SGA1a;A8JFY9_CHLRESerineglyoxylateaminotransferaseOS=ChlamydomonasreinhardtiiGN=SGA1a;D8U556									
A8JHB4	1	1	0.853516	0.84571	1.090964	1.17062	0.873176	1.119584	0.879427
B0JWW6	1	1	1.023542	1.315258	1.301959	0.906492	1.076405	0.985855	0.948425
B6E5W6;I0Z5K3	1	1	0.951361	1.164139	1.101265	1.225166	1.138143	1.108552	1.083288
C1MYV3;E1ZLQ3	1	1	0.977842	0.92444	1.01375	0.956018	0.984548	0.858827	0.931846
C1N9S5	1	1	1.002532	0.947548	0.966582	0.965749	1.02568	1.053385	1.064696
CON__P13645	2	1	1.076447	1.100705	1.432386	1.340294	0.887486	1.160905	1.117775
D8THK6;A8HXS9	1	1	0.935741	1.058762	0.743054	0.853522	0.777879	0.884342	0.695925
D8TJ31;A8I980	1	1	1.121068	1.101703	1.051626	1.113182	0.996552	1.129037	1.099129
D8TJY9;A8IRK4	1	1	0.948785	0.775264	1.048048	1.255121	1.071393	0.853164	1.039231
D8TK12;A8IE23;E1Z520;D8LQV8;A4S521;K8ENB0;B6E5W6;I0Z5K3	1	1	1.48611	0.410639	0.561364	0.764369	1.045294	0.732806	0.748117
D8TKA7;A8IMK1;C1NAA3	2	1	1.026342	0.771882	0.60985	0.864997	0.893218	0.86543	0.941836

D8TM08	1	1	1.041581	1.025576	0.915155	0.812529	0.676846	0.927346	0.892613
D8TPD5;A8IL08;K8EJA2	1	1	0.918025	1.166328	1.233127	0.834334	0.867977	1.173713	1.082694
D8TT41;A8I7T8;A8I7S9	1	1	0.85073	1.223418	1.126879	1.133693	1.228957	1.005191	1.063897
D8TUW7;A8IAT4;I0YXF1;C1N3E5;E1ZG55	1	1	0.965604	1.233253	1.140006	1.016648	1.008317	1.147406	1.121182
D8TV46	1	1	0.978653	1.329397	1.218968	1.366817	1.27008	1.098911	0.943104
D8TYV7	1	1	1.189414	1.610517	1.395139	0.509389	0.453673	1.137462	1.120312
D8U1R3;E1ZP98;I0Z1A5;B0JJ69	2	1	1.052725	0.88743	0.938872	1.367737	1.13244	0.835848	0.845153
D8U3K8;Q5NKW4	1	1	1.061683	1.142268	1.136416	1.269109	0.797222	0.864993	0.786288
D8UC42;A8IA45;K8EK64;I0Z9U5	1	1	0.882419	1.076004	0.978834	0.93426	1.105444	1.52941	1.792807
D8UEA2;A8JFV6	1	1	0.879636	0.845974	0.834761	0.620945	0.901887	0.962044	0.990742
E1ZD58;I0YR87	1	1	1.051241	1.004994	1.102615	0.978608	1.092229	0.979162	1.108714
I0YKI7;P93662;D8TII9;D7G5X8;A4S9E0;Q8RY44	1	1	0.885534	1.004449	0.892719	0.8862	0.950722	1.132163	1.227607
I0YRY7	1	1	0.621998	0.390351	0.422298	0.54317	0.474015	0.485328	0.506659
I0Z028	1	1	0.937749	1.051785	1.125487	1.271484	1.015811	0.778333	0.879379
I0Z401;E1Z7W6;	1	1	0.907262	1.422514	1.35173	1.41181	1.057689	1.066882	1.000623

B0JJU1;D8LB71;A8JHB4									
K8EQC7;C1MZG8;G4WUV8;G3LTV5;A8J8Y1;A4S6H8	1	1	0.93761	0.72289	0.785588	0.677273	1.084698	1.119222	1.021777
P06007;Q1KVW6;Q4JLT1;K8FE34;K7NRG3;F2YGQ0;E9NPS3;D0FXW8;A0A1C8XRK9	1	1	0.785504	0.62868	0.716806	0.736701	0.759298	0.706655	0.700789
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3	1	1	1.002208	1.487932	1.468196	1.292856	1.871486	1.653946	1.650796
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;F2YGQ9;Q1KVU0	1	1	0.848012	0.977506	0.943243	0.894658	0.974487	1.160314	1.05351
Q1KVS9;A0A120N1C6;A0A172BZR9;A0A110B8L5;A0A0X8XG29;P17746;A0A110B817;A0A120N1C5	1	1	0.874741	0.732588	0.921701	0.866533	1.049332	1.045323	1.051964
A0A0C4K0H7	1	1	0.943975	0.482369	0.884671	1.703644	2.527646	0.722119	0.954612
A4RQS5;C1MLH6	1	1	1.023044	0.797527	0.877099	0.778252	0.777893	0.739253	0.865748
A8HW56;D8TIS4;E1Z5R3;C1MLD8;A4RRG4;K8E	1	1	0.942407	0.742123	0.718557	0.723805	0.768136	0.882006	0.857533

910;I0YZZ5									
A8ISB0;A8ISA9;D8TSY0;D8TK58;E1ZQX7;A8IEE5	1	1	0.979338	1.045782	1.187912	1.279753	1.067122	0.956921	0.858537
A8IW00;D8TM93;A8IVZ9;D8TM95;I0YYN3	1	1	0.945809	0.816722	1.0043	0.898982	1.134597	0.925606	1.039547
A8IXE0;E1ZSI5;I0YKP7	1	1	1.039172	1.275353	1.265324	1.317971	1.193783	1.094812	1.133517
A8J1G8	1	1	1.108034	0.934069	0.991338	1.159916	1.086674	0.993211	0.979835
A8J680;D8TNW2;A8J682	1	1	1.039119	1.119491	1.070769	0.978681	1.012303	1.522006	1.307836
A8J841_CHLREHydroxymethylpyrimidinephosphatesynthaseOS=ChlamydomonasreinhardtiiGN=THICb;D8U387	1	1	1.074971	1.410877	1.467342	1.251414	1.111281	1.246611	1.05368
A8J906;D8TIJ1	1	1	1.011711	0.868804	1.01442	1.019531	0.902228	0.955079	0.975545
B0JM87	1	1	0.865277	0.769713	0.869121	0.723955	0.756682	0.880141	0.991719
C1MXS6	1	1	1.006903	0.74013	0.617694	0.570991	0.417837	0.515525	0.543143
D7FK90;D8LI58	1	1	1.440379	0.688044	0.615473	0.563851	0.496606	0.483291	0.549645
D8TKA7	1	1	1.078727	0.793907	0.784786	1.386654	1.140643	0.77392	0.80518
D8TPM9;A8ICT1	1	1	1.009387	0.638048	0.796458	0.756612	0.939259	0.70545	0.784311
D8TRG5	1	1	1.098013	1.129125	0.957736	1.031709	1.171232	1.405534	1.237521

D8TZZ8;A8JIB7;K8FER3	1	1	1.16998	1.450528	1.361347	1.390596	0.975694	1.36958	1.075368
D8UBP2	1	1	0.926997	0.962508	0.893626	0.979947	0.915958	1.008143	0.967357
D8UC92	1	1	0.851748	1.05948	1.049468	0.93419	0.77171	0.867262	0.858201
D8UHN1;E1ZLJ5	1	1	1.134588	1.2759	1.070026	0.992937	0.902961	1.194613	1.109081
E1Z349;I0Z036	1	1	1.002972	0.930662	0.960869	0.933305	1.012741	0.795619	0.845248
E1Z5I7;A8IZZ4;D8U995;I0Z7P3;A4S861	1	1	1.063556	0.892276	1.022293	0.786566	0.820696	1.081353	1.059273
E1ZCK4	1	1	1.260859	1.151411	0.993315	1.193119	1.07666	1.07427	1.005477
E1ZFD0	1	1	0.995147	0.992941	0.969086	0.968602	0.95836	0.985217	0.958931
E1ZI27;I0YWG6	1	1	0.948761	0.855124	0.920349	0.907728	0.966986	1.120183	1.06963
I0YLA9	1	1	1.069611	0.900018	1.037796	1.100043	0.871878	0.986828	1.070346
I0YX80;D8UIY5	1	1	1.092094	1.215129	1.110564	1.21112	1.416797	1.378458	1.421242
I0YXL9	1	1	0.805052	0.858914	0.82073	0.737615	0.817485	0.84439	0.770769
I0Z1E7	1	1	1.026236	0.893699	0.938327	1.0723	1.02371	1.473195	1.261913
I0Z9U5;E1ZH03	1	1	1.103302	1.304796	1.288166	1.402876	1.185312	1.152387	1.017873
K8EDQ7	2	1	0.866931	1.367384	1.26491	0.801689	0.827512	0.9346	0.911377
P02769;CON__P02769;CON__P02768-1	1	1	1.074587	1.324426	1.067096	1.50313	1.209043	0.986279	0.94703
Q1HVA2;E1ZT2	1	1	0.920553	1.198315	1.202546	1.538292	1.351673	1.180745	1.444801

0;D8U9J4;A8HP84;Q1HVA0;B1PL92;I0YMA8;B0JHH3;K8E991;A4RQR7;Q20FC5									
Q1KVU8;Q2TGZ5;P07753;K7NSM7;D0FY08;F2YGK0;B5AID8;K8F0V5;E9NPR9;C1KRD0;P12719;A0A097PB29;A0A023SZ91;A0A023SYH6	1	1	0.63981	1.394278	2.116254	1.141529	1.417687	1.529189	1.19717
Q84X75;E1ZFR4;D8TK78	1	1	0.947035	1.561915	1.526697	1.087241	1.173146	1.346483	1.278516
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;M1VNR7;M1VK51;M1VK44;M1VEJ4;Q3S3F0;S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6;A0A0X8XG25;H6X2F8;W6AAY4;W6AAZ3;E9NPX3;F2YGL1;A0A172C918;P00877;A0A0A0QZL6;H6V738;A0A0A0R1Z2;H6V743;H6V741;H6V742;H6V739;H6V740;H6V7	1	1	1.07157	1.184772	0.997851	0.665265	0.773678	1.285348	1.331136

37;H6V736;H6V735;H6V734;H6V733;F8RPR6;M1VNS0;M1VNR5;M1VK48;M1VEI5;M1V8T6;M1V8T3;M1UZC6;M1UZC1;Q2I3M2;Q2I3M1;R4IUI5;Q2I3L0;U6A3V6;Q3S3F2;Q3S3E9;Q3S3E8;Q3S3E6;Q3S3E5;Q3S3E4;Q3S3E3;Q2I3M8;Q2I3M7;Q2I3M6;Q2I3M5;Q2I3M3;Q2I3L9;Q2I3L8;Q2I3L7;Q2I3L6;Q2I3L5;Q2I3L2;Q2I3L1;Q2I3K9;Q2I3K8;Q2I3K7;Q2I3K6;Q2I3K5;Q2I3K4;Q2I3K3;Q2I3K2;Q2I3K1;Q2I3K0;Q2I3J9;Q2I3J8;O65776;M1J7Z0;Q1XIR3;Q1XIR2;Q1XIR1;Q6QNV1;A0A0E3JP63;Q2TGZ2;K7NSN7;D0FXZ7;A0A1C8XRQ3;A0A110B8J5;M1VEI8;Q2I3J7;Q8HD99;W6A241									
A0A125YZR4	1	1	1.288192	1.553731	1.482231	0.196489	0.329189	1.091056	0.900395
A0A172C1L3	1	1	0.850395	0.934053	0.857074	1.085716	0.807648	0.833532	0.76264

A8I972	2	1	1.117756	0.610035	0.765238	0.916272	1.203392	0.938587	0.989353
A8IWJ3;A4S410	1	1	0.974916	0.961285	0.795578	1.270565	0.968893	0.795179	0.722261
A8J786;E1Z3T4;D8UEQ8	1	1	1.084838	1.17298	1.180484	1.070992	0.98185	1.001375	0.861146
A8JFJ2	1	1	0.999001	1.073731	1.039771	1.098193	0.914079	0.856169	0.876105
A8JJG8;A8JJV5;A4S1C9;K8EHQ7;C1MHL2;A8JJN6;D8UMG1;A8JJS0;A8IJR6;A8JIN6;A8JDH1;A8JDE1;A8JDC9;A8JDC0;A8IR79;A8IR69;A8IJS4;A8HWX5;A8HWX1;A8HWE3;A8HV98;D8TP10;D8TNF1;D8UDT7;A8IW84;A8IW75;D8U9Y1;D8TZB9;A8HSB2;D8TM85;D8TI76;D8TIA7;D8TI79;K8EFG9;K8EZ76	1	1	1.028193	1.072832	1.069324	0.92791	0.943723	1.011125	1.125501
D8TJ56	1	1	0.858717	1.034504	1.34563	0.818021	1.064235	1.212089	1.442227
D8TKE8;A8IMZ5;F2YGP6;E9NPX7	1	1	1.061336	1.073918	1.03383	1.107197	1.003217	1.066238	0.99392
D8TLB0;A8J1U1	1	1	1.235785	0.712362	0.71044	1.100478	0.826938	0.812374	0.782501
D8TRZ4	1	1	1.205845	1.03796	1.006163	1.321596	0.913003	1.182407	0.868794

D8TTD9;A8HP06;C1N7J8	1	1	0.994153	1.192829	1.093	1.156217	0.97975	1.235842	1.182555
D8TZU3;Q6SA05	1	1	0.882757	1.115675	1.397731	1.262952	0.943553	1.324632	1.309808
D8TZZ8;A8JIB7;E1ZRV3	2	1	1.27096	0.397154	0.561621	0.64129	0.802666	0.6086	0.867946
D8U1R3	1	1	0.950597	1.147252	1.24152	1.058069	0.977373	1.296917	0.929339
D8U3U0;A8JFT3;K8EC93	1	1	1.095902	1.347166	1.175405	1.562142	1.355703	0.976046	1.028077
D8UBQ8;Q9LLL6	1	1	1.035381	0.283094	0.382333	0.563774	0.726392	0.63017	0.670872
D8UI88	1	1	0.980245	0.650192	0.621005	0.619637	0.671436	0.774399	0.846895
E1Z349	1	1	1.10272	1.389948	1.229699	0.890299	1.658569	1.475806	1.661362
E1ZQ02;I0Z789	1	1	1.074425	0.857192	1.003343	0.834164	1.022423	0.942978	1.041902
G4WUV9	1	1	0.974615	1.098216	1.052912	1.001756	1.292564	1.02066	0.94187
I0YKI7;A8JEU4	1	1	0.745052	0.857227	1.265586	2.010112	1.214634	0.82245	0.787046
K8EQC7;C1MZG8;I0Z9Y9;D8TIF4	1	1	1.129155	1.225178	1.117361	1.282316	1.059314	1.096529	1.025375
Q1KVU0	1	1	1.026089	0.768778	0.802987	0.965526	1.076665	0.940301	0.940576
A8HZZ1;D8TM26;I0YN25;E1ZCK4	1	1	0.733587	1.047602	1.080145	0.742364	0.728761	1.075566	1.074545
A8IA39	1	1	1.053121	1.113618	1.202697	1.1464	0.974742	1.060583	0.968103

A8IB25;D8TKV1	1	1	0.888147	0.790932	0.776336	1.49307	1.072018	0.774971	0.757733
A8J1T4	1	1	1.130359	0.861811	0.893533	1.103486	0.923582	0.860039	0.855683
D8TYV7;I0YU56; A8JC04	1	1	0.93286	0.957282	1.096301	0.872732	0.941945	1.142845	1.012729
K8F9G7	1	1	1.126201	0.958924	0.846644	1.271892	1.038031	0.917561	0.861759
Q75VY8;D8UAY 7	1	1	0.920464	1.259477	0.937279	1.134744	1.149342	0.864266	0.945758
		1	0.969103	0.701868	0.732435	0.747676	1.243901	0.908276	1.138503

SECTION II – PHENOTYPES COMPARISONS

ITRAQ#1: +2H EXPOSURE

ASTM VS CONTROL

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTS	FOLD CHANGE	P
E1ZBK2	Putative uncharacterized protein	Chlorella variabilis	1	1.85	2.81E-04
D8U4Q1	Putative uncharacterized protein	Volvox carteri f. nagariensis	2	1.72	1.68E-03
I0YQ64	Catalase	Coccomyxa subellipsoidea (strain C-169)	1	1.65	2.48E-02
I0Z028	Vitamin B6 biosynthesis protein	Coccomyxa subellipsoidea (strain C-169)	1	1.54	1.52E-03
A4S0V1	Uncharacterized protein	Ostreococcus lucimarinus (strain CCE9901)	1	1.51	1.97E-04
E1ZBK2	Eukaryotic elongation factor 1 alpha	Ostreococcus lucimarinus (strain CCE9901)	1	1.50	5.66E-03
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.47	1.96E-04
D8UI03	HSP70bf	Volvox carteri f. nagariensis	1	1.41	5.15E-03
A0A0S1LH61	Peptidylprolyl isomerase	Scenedesmus sp. FKBP	1	1.37	1.32E-03
D8TV46	Putative uncharacterized protein	Volvox carteri f. nagariensis	1	1.37	2.08E-02
Q1KVT0	ATP synthase subunit beta	Scenedesmus quadricauda	2	1.33	1.70E-04
D8U4Q1	Transketolase	Chlorella variabilis	1	1.31	2.95E-02
P06007	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	1.30	3.47E-03
E1ZBK2	Eukaryotic translation elongation factor 1 alpha 1	Chlamydomonas reinhardtii	3	1.29	1.41E-03
E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea (strain C-169)	1	1.27	3.95E-04

D8U1R3	ATP-dependent Clp protease ATPase subunit	Microcystis aeruginosa (strain NIES-843)	2	1.23	3.09E-02
A8IQU3	ATP synthase subunit beta	Chlamydomonas reinhardtii	1	1.23	1.02E-02
A0A0C4K0H7	SBP protein	D. tertiolecta	2	1.22	9.02E-04
D8UFR3	40S ribosomal protein S12	Volvox carteri f. nagariensis	3	1.22	3.67E-03
D7FK90	Molecular chaperones HSP70/HSC70, H...	Ectocarpus siliculosus (Brown alga) (Conferva siliculosa)	1	1.22	2.51E-04
A8IZZ4	Bi-ubiquitin	Chlamydomonas reinhardtii	1	1.21	6.76E-03
Q1KVT0	ATP synthase subunit beta, cyanelle	Cyanophora paradoxa	1	1.21	1.10E-05
I0YRY7	14-3-3 protein	Coccomyxa subellipsoidea (strain C-169)	1	1.20	2.14E-03
P06007	Photosystem II D2 protein	Bathycoccus prasinos	1	1.20	1.98E-02
Q1KVS9	Elongation factor Tu, chloroplastic	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.20	1.85E-02
Q1KVT0	ATP synthase subunit beta, chloropl...	Chlamydomonas reinhardtii	3	1.19	2.35E-05
Q1KVV6	Photosystem II CP47 reaction center protein	Dunaliella tertiolecta	1	1.19	6.08E-04
A0A1B0VE51	Superoxide dismutase	Scenedesmus acutus	1	1.18	2.04E-04
A8IQU3	ATP synthase subunit beta	Bathycoccus prasinos	1	1.18	6.20E-03
A0A172C1L3	Elongation factor Tu	Scenedesmus sp. CCMA_UFSCar 088	1	1.17	1.71E-02
B0JXA3	Phycocyanin beta subunit	Microcystis aeruginosa (strain NIES-843)	1	1.16	1.16E-02
A8JBG5	Flavoprotein	Chlamydomonas reinhardtii	1	1.15	2.89E-02
A8IZU0	Heat shock protein 70C	Chlamydomonas reinhardtii	1	1.15	6.90E-04
A8IX80	Acetohydroxyacid dehydratase	Chlamydomonas reinhardtii	2	-1.16	2.22E-04

Q42690	Fructose-bisphosphate aldolase	Chlorella variabilis	1	-1.23	9.36E-03
A8IW00	Glutamine synthetase	Chlamydomonas reinhardtii	1	-1.27	4.52E-03
D8TK12	Glucose-6-phosphate isomerase	Volvox carteri f. nagariensis	1	-1.34	1.80E-02
Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	-1.35	1.26E-04
A8JEU4	Heat shock protein 70A	Chlamydomonas reinhardtii	1	-1.36	1.11E-03
I0YZ27	Glyoxalase I	Coccomyxa subellipsoidea (strain C-169)	1	-1.41	1.70E-02
K8EHR6	PsaD, PSI-D, subunit II, photosystem I protein	Ostreococcus lucimarinus (strain CCE9901)	1	-1.51	1.39E-02
A4RTP0	Malate dehydrogenase	Ostreococcus lucimarinus (strain CCE9901)	1	-1.51	6.66E-03
Q1KVT0	ATP synthase subunit beta, chloropl...	Tetradesmus obliquus (Acutodesmus obliquus)	1	-1.56	2.22E-02
Q84RL9	Enolase	Dunaliella salina (Protococcus salinus)	2	-1.58	9.49E-04
C1MNA2	Predicted protein	Micromonas pusilla (strain CCMP1545) (Picoplanktonic green alga)	1	-1.65	2.09E-02
D8TZD7	Chaperonin 60B2	Chlamydomonas reinhardtii	1	-1.79	2.85E-02
Q8VXQ9	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	Coelastrella vacuolata (Chlorella fusca var. vacuolata)	1	-2.00	1.19E-02
Q42690	Fructose-bisphosphate aldolase 1, chloroplastic	Chlamydomonas reinhardtii	2	-2.17	1.87E-05

DW$_{PLK}$ VS. CONTROL

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
K8EHR6; A4S7X2; C1N6J0	Uncharacterized protein	Bathycoccus prasinos	1	1.997	5.900E-03
Q1KVV6	Photosystem II CP47 reaction centre protein (PSII 47 kDa protein) (Protein CP-47)	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.844	7.747E-06
Q2TGZ4; D0FY05; A0A1C8XRM6; P37255	Photosystem II CP47 reaction centre protein (PSII 47 kDa protein) (Protein CP-47)	Dunaliella tertiolecta	1	1.676	4.377E-03
Q1KVU8; F2YGK0	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.571	1.772E-03
A8JJG8	Histone H2B	Chlamydomonas reinhardtii	1	1.453	3.823E-03
Q1KVY2; E9NPS2; P10898; K7NU72; D0FXY3; A0A1C8XRL7	Photosystem II CP43 reaction centre protein (PSII 43 kDa protein) (Protein CP-43)	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.446	2.688E-02
Q1KVY2; E9NPS2; P10898; K7NU72; D0FXY3; A0A1C8XRL7; W8E1S1; B0JR68; F2YGQ1	Photosystem II CP43 reaction centre protein	Dunaliella parva	1	1.380	2.563E-03
D8U3K8; Q5NKW4	Photosystem I reaction centre subunit II, 20 kDa (Photosystem I subunit)	Chlamydomonas reinhardtii	1	1.377	1.355E-02
A8IZZ4; D8U995; D8U547	Bi-ubiquitin	Chlamydomonas reinhardtii	1	1.329	7.937E-03
A8J1G8	40S ribosomal protein S6	Chlamydomonas reinhardtii	1	1.324	2.860E-02
P06007	Photosystem II D2 protein (PSII D2 protein) (EC 1.10.3.9) (Photosystem Q(A) protein)	Chlamydomonas reinhardtii	1	1.264	1.047E-02
Q1KVX3; K7NSN1; A0A1C8XRP4	Cytochrome b559 subunit beta (PSII reaction centre subunit VI)	Tetradesmus obliquus (Acutodesmus obliquus)	2	1.248	3.891E-02
A0A1B0VE51	Superoxide dismutase (EC 1.15.1.1)	Scenedesmus acutus	1	1.186	9.888E-03
I0YP36	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	Coccomyxa subellipsoidea (strain C-169) (Green microalga)	1	1.000	1.562E-02
E1Z6L2	Uncharacterized protein	Chlamydomonas reinhardtii	2	-1.096	1.654E-02
D8U1T0	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.127	1.738E-02
S4VNM6; H6X2F8; H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.208	8.821E-03
D8U4Q1	Uncharacterized protein	Chlamydomonas reinhardtii	2	-1.305	8.018E-03
S4ULQ5	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	Scenedesmus acutus	1	-1.399	1.733E-03
S4VNM6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	4	-1.670	3.884E-04

DWFLOC – CONTROL

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVY3	Photosystem I P700 chlorophyll a apoprotein A1	Tetradesmus obliquus (Acutodesmus obliquus)	1	2.28	2.36E-02
Q1KVV6; E9NPV5	Photosystem II CP47 reaction center protein	Coccomyxa subellipsoidea (strain C-169)	1	2.14	4.16E-04
Q1KVV6; Q2TGZ4; D0FY05; A0A1C8XRM6; P37255	Photosystem II CP47 reaction center protein	Dunaliella tertiolecta	1	1.78	4.30E-04
K8EHR6; A4S7X2; C1N6J0	Uncharacterized protein	Bathycoccus prasinos	1	1.74	3.80E-03
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.62	1.10E-06
D8U3K8; Q5NKW4	Chaperonin 60A	Chlamydomonas reinhardtii	24	1.38	2.78E-02
I0YS06; H2ELS9; D8TSK8; A8JHQ7; C1MIT8	GTP-binding protein YPTC1	Coccomyxa subellipsoidea	1	1.37	1.69E-02
D8UI88	Uncharacterized protein	Volvox carteri f. nagariensis	3	1.33	6.24E-03
E1ZQY4	40S ribosomal protein S5	Chlorella variabilis	2	1.31	5.22E-03
A8IZZ4; D8U995; D8U547	Bi-ubiquitin	Chlamydomonas reinhardtii	4	1.30	1.75E-03
I0YUW3	Elongation factor 2	Coccomyxa subellipsoidea	2	1.29	1.85E-02
Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii	1	1.23	1.75E-02
Q1KVT0; P06541; Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus, (Acutodesmus obliquus)	1	1.23	3.45E-03
E1ZD58; I0YR87	Cysteine synthase	Chlorella variabilis	1	1.22	7.76E-03
Q1KVY2; E9NPS2; P10898; K7NU72; D0FXY3; A0A1C8XRL7; W8E1S1; B0JR68; F2YGQ1	Photosystem II CP43 reaction center protein	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.21	6.12E-03
D8TZZ8; A8JIB7; E1ZRV3	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.21	9.19E-03
A8IWQ7; D8UEY8	Predicted protein	Chlamydomonas reinhardtii	1	1.20	9.97E-03
P06007; Q1KVW6; Q4JLT1	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	1.19	1.01E-02
A8J5P7; D8TNA2	Ubiquinol:cytochrome c oxidoreductase 50 kDa core 1 subunit	Chlamydomonas reinhardtii	1	1.15	6.89E-03
Q1KVX3; K7NSN1; A0A1C8XRP4	Cytochrome b559 subunit beta	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.14	1.50E-02
S4VNM6; H6X2F8; H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus sp. LU1	1	-1.11	1.49E-02
S4VNM6; H6X2F8; H6X2P3; A0A110B8J4; A0A110B723	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus obtusus	2	-1.15	1.76E-02
D8UBP2	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.15	1.82E-02

D8TUP1; A8J7F6	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Chlamydomonas reinhardtii	2	-1.22	4.55E-03
I0Z401	Ferredoxin-dependent glutamate synthase	Coccomyxa subellipsoidea	4	-1.23	2.28E-03
G4WUV9	Chloroplast ATP synthase gamma chain protein	Dunaliella salina	4	-1.25	1.99E-02
S4VNM6; H6X2F8; H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.30	7.21E-05
K8F9G7	Uncharacterized protein	Bathycoccus prasinos	2	-1.33	1.71E-02
D8TUP1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Volvox carteri f. nagariensis	1	-1.41	2.83E-02
S4VNM6; H6X2F8; H6X2P3; A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus obtusus	1	-1.44	3.71E-03
A8JEU4; E1ZQV2	Heat shock protein 70A	Chlamydomonas reinhardtii	2	-1.45	8.98E-03
S4ULQ5	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	Scenedesmus acutus	1	-1.46	1.65E-02

DW_{PLK} vs ASTM

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVT2; E9NPX5; D1J798	Cytochrome b6	Tetradesmus obliquus (Acutodesmus obliquus)	1	2.35	2.41E-02
E1ZBK2	Uncharacterized protein	Chlorella variabilis	1	2.07	1.61E-03
D8U4Q1; E1ZGR1; A8IAN1; K8ERB6; I0YJZ4	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.95	2.39E-02
Q1KVV6	Photosystem II CP47 reaction centre protein	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.59	9.64E-03
Q1KVU8; F2YGK0	Photosystem II protein D1	Tetradesmus obliquus (Acutodesmus obliquus)	1	1.45	2.29E-04
A4SB22; K8EL02; C1MWS0; B5A517	Uncharacterized protein	Ostreococcus lucimarinus (strain CCE9901)	1	1.43	1.43E-02
K8EHR6; A4S7X2; C1N6J0	Uncharacterized protein	Bathycoccus prasinos	1	1.36	6.64E-03
D8UFR3; A8J9T0	40S ribosomal protein S12	Volvox carteri f. nagariensis	3	1.34	8.99E-03
D8UI03; E1ZE03; A8HYV3; Q8VY41; Q9M452; I0Z190	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.32	4.66E-03
E1ZBK2; D8TNN3; D8THW4;	Eukaryotic translation elongation factor 1 alpha 2	Volvox carteri f. nagariensis	1	1.29	8.03E-04
A8IX80; D8UGB5	Acetohydroxyacid dehydratase	Chlamydomonas reinhardtii	2	-1.15	1.01E-03
D8TV46; A8IRQ1; E1Z7C4	Ribose-5-phosphate isomerase (EC 5.3.1.6)	Chlamydomonas reinhardtii	2	-1.16	6.30E-03
A0A0S1LH61	Peptidylprolyl isomerase (EC 5.2.1.8)	Scenedesmus sp. FKBP	1	-1.16	3.25E-03
D8TV46; A8IRQ1	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.16	1.77E-02
D8UBP2	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.18	2.07E-02
A0A0C4K0H7; I0YIH9	SBP protein (EC 3.1.3.37)	Dunaliella tertiolecta	2	-1.19	7.59E-03
P06007; Q1KVW6	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	-1.21	1.42E-02
S4ULQ5	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	Scenedesmus acutus	1	-1.48	2.16E-03
Q1KVT0; P06541; Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus (Acutodesmus obliquus)	3	-1.78	5.44E-05
A8JBG5	Flavoprotein	Chlamydomonas reinhardtii	1	-2.06	1.19E-02
S4VNM6; H6X2F8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-2.21	1.46E-06

DW_{FLOC} VS ASTM

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVT0; P06541; P48081; A0A097PBH6; Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	1.70	1.15E-07
D8U4Q1; E1ZGR1; A8IAN1; K8ERB6; I0YJZ4	Uncharacterized protein	Volvox carteri f. nagariensis	2	1.67	1.91E-02
A8IQU3; D8TRA2; K8EN95; A4RSS5; C1MKD5; I0YLJ1	ATP synthase subunit beta	Chlamydomonas reinhardtii	2	1.58	1.11E-02
Q1KVV6	Photosystem II CP47 reaction centre protein	Tetradesmus obliquus	1	1.39	1.39E-04
I0YUW3	Elongation factor 2	Coccomyxa subellipsoidea	2	1.36	3.59E-03
D8UFR3; A8J9T0	40S ribosomal protein S12	Volvox carteri f. nagariensis	1	1.35	7.37E-04
E1ZBK2; D8TNN3; D8THW4; A8HX38	Uncharacterized protein	Chlorella variabilis	1	1.33	1.08E-03
I0YP36	Isocitrate dehydrogenase [NADP]	Coccomyxa subellipsoidea	1	1.25	1.29E-02
A8IQU3; D8TRA2; E1ZS63; I0YLJ1	ATP synthase subunit beta	Chlamydomonas reinhardtii	1	1.22	1.95E-02
E1ZBK2; D8TNN3; D8THW4; A8HX38; A4S6B6; K8F4B8; C1MZI5; C1MT59	Eukaryotic translation elongation factor 1 alpha 2	Volvox carteri f. nagariensis	5	1.22	4.84E-04
D8TTF7	Plastid acyl-ACP desaturase	Volvox carteri f. nagariensis	2	1.21	1.95E-02
D8UI03; E1ZE03; A8HYV3; Q8VY41; Q9M452; I0Z190	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.21	1.58E-02
A8IQU3; D8TRA2	ATP synthase subunit beta	Chlamydomonas reinhardtii	4	1.20	1.22E-03
A8ISB0; A8ISA9; D8TSY0; D8TK58; E1ZQX7; A8IEE5	Cysteine synthase	Chlamydomonas reinhardtii	1	1.16	1.77E-02

D8U4Q1	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.13	1.38E-02
A0A097PB89	ATP synthase subunit alpha	Cyanophora paradoxa	1	1.11	2.95E-02
Q84RL9	Enolase	Dunaliella salina	1	-1.13	2.96E-03
P06007; Q1KVW6	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	-1.13	6.37E-03
S4VNM6; H6X2F8; H6X2P3; A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.14	1.72E-02
A8IX80; D8UGB5	Acetohydroxyacid dehydratase	Chlamydomonas reinhardtii	1	-1.15	6.57E-03
A8IZU0; D8TMR1; B7TJI1	Heat shock protein 70C	Chlamydomonas reinhardtii	1	-1.19	2.04E-03
A0A0S1LH61	Peptidylprolyl isomerase	Scenedesmus sp. FKBP	2	-1.19	4.66E-03
I0YQ64; A8J537	Catalase	Coccomyxa subellipsoidea	3	-1.21	1.30E-02
E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea	1	-1.22	5.47E-04
Q1KVT0	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	-1.23	5.47E-04
I0Z401	Ferredoxin-dependent glutamate synthase	Coccomyxa subellipsoidea	1	-1.24	3.22E-03
A4S0V1	Uncharacterized protein	Ostreococcus lucimarinus (strain CCE9901)	1	-1.25	3.64E-03
Q42690; D8TKY4; I0YN66; E1ZQQ5	Fructose-bisphosphate aldolase 1, chloroplastic	Chlamydomonas reinhardtii	1	-1.25	1.09E-05
A8IW00; D8TM93; A8IVZ9; D8TM95; I0YYN3	Glutamine synthetase	Chlamydomonas reinhardtii	3	-1.25	6.38E-03
A0A0C4K0H7; I0YIH9	SBP protein	Dunaliella tertiolecta	2	-1.26	2.46E-03
G4WUV9	Chloroplast ATP synthase gamma chain protein	Dunaliella salina	4	-1.27	5.97E-03
D7FK90; D8LI58; D7FZN2; E1ZQV2	Molecular chaperones HSP70/HSC70, HSP70	Ectocarpus siliculosus	1	-1.28	1.63E-03

	superfamily	(Brown alga) (Conferva siliculosa)			
D8UBP2	Uncharacterized protein	Volvox carteri f. nagariensis	2	-1.28	3.19E-03
Q1KVY2	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	2	-1.36	3.40E-02
Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	-1.42	1.18E-04
K8F9G7	Uncharacterized protein	Bathycoccus prasinos	1	-1.42	1.48E-02
S4VNM6; H6X2F8; H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.48	1.18E-05
A8JCY4; D8U593; I0YSE8	Fructose-bisphosphate aldolase	Chlamydomonas reinhardtii	2	-1.49	3.31E-02
A8JBG5	Flavoprotein	Chlamydomonas reinhardtii	1	-1.50	2.32E-02
S4ULQ5	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	Scenedesmus acutus	1	-1.55	1.22E-02
D8TUP1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Volvox carteri f. nagariensis	1	-1.72	2.03E-02

DW_{FLOC} VS. DW_{PLK}

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVT0; P06541; Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	3	1.60	1.55E-03
S4VNM6; H6X2F8; H6X2P3; A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	1.50	1.81E-03
Q1KVT0; P06541; P48081; A0A097PBH6; Q8HDG4	ATP synthase subunit beta, chloroplastic	Chlamydomonas reinhardtii	1	1.36	1.38E-02
D8U1T0	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.22	1.29E-02
A8HYU5; C1N037	S-adenosylmethionine synthase	Chlamydomonas reinhardtii	1	1.20	3.57E-02
D8TTF7	Plastid acyl-ACP desaturase	Volvox carteri f. nagariensis	1	1.19	2.93E-02
S4VNM6; H6X2F8; H6X2P3; A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit	Scenedesmus sp. LU4	1	-1.20	1.32E-02
S4VNM6; H6X2F8; H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit	Scenedesmus sp. LU1	2	-1.25	1.64E-02
Q1KVU8; F2YGK0	Photosystem II protein D1	Tetradesmus obliquus	1	-1.28	3.86E-03
A8JJG8; A8JJV5; A4S1C9	Histone H2B (Fragment)	Chlamydomonas reinhardtii	1	-1.31	8.31E-04
G4WUV9	Chloroplast ATP synthase gamma chain protein	Dunaliella salina	1	-1.32	1.00E-02
D8UF20; A8IWJ5; E1ZAJ1	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.55	2.39E-02
A8IYP4; D8TRR7; E1ZF27	Phosphoribulokinase	Chlamydomonas reinhardtii	1	-1.68	7.57E-03

iTRAQ#2: +20h Exposure

ASTM vs. Control

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1HVA2;E1ZT20;D8U9J	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	Chlamydomonas reinhardtii	1	2.15	2.07E-02
Q1KVY2	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	2.03	7.37E-04
P06007;Q1KVW6;Q4JLT1	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	1.78	1.34E-02
C1N5S1	Predicted protein	Micromonas pusilla	1	1.75	1.77E-02
Q1KVU8;F2YGK0	Photosystem II protein D1	Tetradesmus obliquus	1	1.62	5.75E-03
Q1KVU8;Q2TGZ5;P07753	Photosystem II protein D1	Dunaliella tertiolecta	1	1.59	3.00E-02
Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	1.57	3.33E-05
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus	1	1.55	1.27E-03
Q1KVY2;E9NPS2;P10898	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	1.52	2.04E-03
Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii	1	1.44	9.33E-03
I0YL77;E1ZL24;D8TPC8	ADP-ribosylation factor 1	Coccomyxa subellipsoidea	1	1.39	1.97E-03
D8TV46	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.37	2.40E-02
A4S0V1	Uncharacterized protein	Ostreococcus lucimarinus (strain CCE9901)	1	1.37	4.49E-03
Q1KVY2;E9NPS2;P10898	Photosystem II CP43 reaction center protein	Chlamydomonas reinhardtii	1	1.32	2.06E-02

E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea	1	1.31	8.60E-08
A8IZU0;D8TMR1;B7TJI1	Heat shock protein 70C	Chlamydomonas reinhardtii	2	1.31	1.57E-02
A0A0S1LH61	Peptidylprolyl isomerase	Scenedesmus sp. FKBP	2	1.29	6.00E-04
A4RTP0	Malate dehydrogenase	Ostreococcus lucimarinus (strain CCE9901)	6	1.29	1.74E-02
Q42690;D8TKY4;I0YN66;E1ZQQ5	Fructose-bisphosphate aldolase 1, chloroplastic	Chlamydomonas reinhardtii	1	1.26	7.64E-08
A8IW00;D8TM93;A8IVZ9;D8TM95;I0YYN3	Glutamine synthetase	Chlamydomonas reinhardtii	1	1.25	2.28E-03
Q1KVT0	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	1.24	4.18E-11
Q1KVT0;P06541;Q8HDG4	ATP synthase subunit beta, chloroplastic	Chlamydomonas reinhardtii	1	1.23	1.30E-02
Q84RL9	Enolase	Dunaliella salina	1	1.21	9.67E-04
A0A0C4K0H7;I0YIH9	SBP protein	Dunaliella tertiolecta	2	1.19	3.08E-03
A0A1B0VE51	Superoxide dismutase	Scenedesmus acutus	1	1.19	3.03E-02
A0A097PB89;D1J797;B0JWV1	ATP synthase subunit alpha	Cyanophora paradoxa	1	1.18	4.73E-03
D7FK90;D8LI58;D7FZN2;I0YNC4;I0YKI7;P93662	Molecular chaperones HSP70/HSC70, HSP70 superfamily	Ectocarpus siliculosus	2	1.18	6.23E-03
D8U477;D7FRY5;I0Z4W2	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, chloroplast	Ectocarpus siliculosus	3	1.15	1.65E-02
D8U5B1;A8JG03	Isopropylmalate dehydratase, large subunit	Chlamydomonas reinhardtii	1	-1.10	2.00E-02
I0YUW3	Elongation factor 2	Coccomyxa subellipsoidea	1	-1.26	3.67E-03
K8F4N5	3-oxoacyl-[acyl-carrier-protein] synthase	Bathycoccus prasinos	1	-1.27	1.56E-02

I0Z401;E1Z7W6;B0JJU1;D8LB71;A8JHB4	Ferredoxin-dependent glutamate synthase	Coccomyxa subellipsoidea	1	-1.28	1.72E-02
E1ZBK2;D8TNN3;D8THW4	Eukaryotic translation initiation factor 1 alpha 1	Volvox carteri f. nagariensis	1	-1.29	3.48E-04
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	1	-1.31	7.80E-04
A4RQS5;C1MLH6	Phosphomannomutase	Ostreococcus lucimarinus (strain CCE9901)	2	-1.33	1.67E-02
P48101;A0A097PB99	Magnesium-chelatase subunit ChlI	Cyanophora paradoxa	1	-1.35	3.55E-02
E1ZBK2	Uncharacterized protein	Chlorella variabilis	3	-1.36	1.59E-02
Q1KVT0;P06541;P48081;A0A097PBH6;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	-1.38	4.09E-03
S4VNM6;H6X2P3;A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.42	3.06E-04
D8U4Q1	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.46	3.85E-03
D8TK12;A8IE23;E1Z520	Glucose-6-phosphate isomerase	Volvox carteri f. nagariensis	1	-1.48	1.98E-02
B0JM87	Uncharacterized protein	Microcystis aeruginosa (strain NIES-843)	1	-1.48	9.01E-03
E1ZBK2;D8TNN3;D8THW4;A8HX38	Eukaryotic translation initiation factor 1 alpha 1	Volvox carteri f. nagariensis	2	-1.55	6.93E-04
D8UFR3;A8J9T0	40S ribosomal protein S12	Volvox carteri f. nagariensis	1	-1.61	9.56E-03
A8IQU3;D8TRA2;K8EN95	ATP synthase subunit beta	Chlamydomonas reinhardtii	1	-1.62	3.12E-02
A8JDV2;D8UIE7	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.67	3.04E-02
A8IQU3;D8TRA2;E1ZS63;I0YLJ1	ATP synthase subunit beta	Chlamydomonas reinhardtii	3	-1.74	1.13E-02
I0YRY7;Q56D00;E1ZIV3	14-3-3 protein	Coccomyxa subellipsoidea	1	-1.86	1.80E-04

D8TJY9;A8IRK4	Sedoheptulose-1,7-biphosphatase	Volvox carteri f. nagariensis	2	-2.54	8.22E-03

DW$_{PLK}$ VS. CONTROL

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q8LRU1;I0YP34;D8TX08	Ferritin	Chlamydomonas reinhardtii	1	2.27	3.37E-03
Q1KVS9;A0A120N1C6	Elongation factor Tu, chloroplastic	Tetradesmus obliquus	1	2.14	6.22E-03
D8UI03;A8HYV3	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.83	9.28E-03
C1N5S1	Predicted protein	Micromonas pusilla	2	1.83	2.26E-02
E1ZQL8	Glutamate-1-semialdehyde 2,1-aminomutase	Chlorella variabilis	1	1.75	2.10E-02
A0A172C1L3;Q1KVS9;A0A120N1C6	Elongation factor Tu	Scenedesmus sp. CCMA_UFSCar 088	1	1.65	5.93E-03
I0YRY7;Q56D00;E1ZIV3	14-3-3 protein	Coccomyxa subellipsoidea	2	1.65	1.93E-04
A0A0X9AMW9;A0A0X9AGK8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	4	1.61	2.94E-05
D8UI03;A8HYV3;E1ZE03	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.51	1.98E-02
S4VNM6;H6X2P3;A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	1.41	2.41E-05
E1Z5I7;A8IZZ4;D8U995;E1Z8A6;D8U547	Bi-ubiquitin	Chlamydomonas reinhardtii	3	1.34	6.33E-04
E1ZMW8	Uncharacterized protein	Chlorella variabilis	1	1.28	1.81E-02
I0YZE5	EF-hand	Coccomyxa subellipsoidea	1	1.22	2.37E-03
A8J6C7;D8TTK4;I0Z5Q8	Membrane AAA-metalloprotease	Chlamydomonas reinhardtii	1	1.20	1.63E-02
E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea	1	1.19	1.24E-02

E1Z6L2	Uncharacterized protein	Chlorella variabilis	1	-1.14	1.58E-03
A0A0C4K0H7	SBP protein	Dunaliella tertiolecta	1	-1.30	8.62E-03
I0YL77;E1ZL24;D8TPC8;A8IL29;A4S5Z2;C1MH11;K8FE75	ADP-ribosylation factor 1	Coccomyxa subellipsoidea	1	-1.31	1.50E-02
A8IQU3;D8TRA2;E1ZS63;I0YLJ1	ATP synthase subunit beta	Chlamydomonas reinhardtii	1	-1.38	5.08E-03
Q84X75;E1ZFR4;D8TK78	CR051 protein (Predicted protein)	Chlamydomonas reinhardtii	1	-1.44	2.05E-02
D8UBQ8;Q9LLL6	Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) (ADP-glucose pyrophosphorylase)	Volvox carteri f. nagariensis	2	-1.53	5.82E-03
A8JEU4	Heat shock protein 70A	Chlamydomonas reinhardtii	1	-1.92	8.90E-03
Q1KVT0;P06541;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	-1.93	2.11E-03
B0JM87	Uncharacterized protein	Microcystis aeruginosa	1	-2.05	1.82E-03
I0YP36	Isocitrate dehydrogenase [NADP]	Coccomyxa subellipsoidea	1	-2.13	4.74E-03
A0A0X9AMW9;A0A0X9AGK8;S4VNM6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	1	-2.16	2.64E-06
C1MXS6	Predicted protein	Micromonas pusilla	1	-2.27	1.80E-02
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU29	1	-3.12	9.97E-09
B7TJI2	Heat shock protein 70B	Dunaliella salina	1	-7.77	2.35E-02

DW_{FLOC} VS. CONTROL

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus	1	3.66	6.22E-05
Q1KVY2	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	2.75	2.99E-05
P37255	Photosystem II CP47 reaction center protein	Chlamydomonas reinhardtii	2	2.57	2.44E-02
Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii	1	2.26	1.99E-03
Q1KVU8;F2YGK0	Photosystem II protein D1 (PSII D1 protein)	Tetradesmus obliquus	1	2.23	9.31E-05
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	2.16	9.28E-04
P06007;Q1KVW6;Q4JLT1;K8FE34	Photosystem II D2 protein	Chlamydomonas reinhardtii	2	2.12	5.05E-03
A8HXL8;E1ZEB1;D8TI16;I0ZA63	Chloroplast ATP synthase gamma chain	Chlamydomonas reinhardtii	2	1.64	1.09E-02
I0Z5X3	Uncharacterized protein	Coccomyxa subellipsoidea	1	1.60	2.31E-02
I0YL77;E1ZL24;D8TPC8;A8IL29;A4S5Z2;C1MH11;K8FE75	ADP-ribosylation factor 1	Coccomyxa subellipsoidea	1	1.57	8.87E-03
Q1KVY2;E9NPS2;P10898;K7NU72;D0FXY3;A0A1C8XRL7	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	1.45	1.10E-02
Q1HVA2;E1ZT20;D8U9J4;A8HP84;Q1HVA0;B1PL92;I0YMA8;Q8VXQ9	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	Chlamydomonas reinhardtii	1	1.43	8.74E-04

A8JJG8;A8JJV5;A4S1C9;K8EHQ7;C1MHL2	Histone H2B	Chlamydomonas reinhardtii	1	1.43	4.99E-03
A8J1G8	40S ribosomal protein S6	Chlamydomonas reinhardtii	1	1.38	2.78E-02
A0A097PB89	ATP synthase subunit alpha	Cyanophora paradoxa	1	1.30	3.80E-03
Q1KVT0;P06541;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	1.25	3.58E-03
C1MYV3;E1ZLQ3;I0YI95;D8UA08;A8JAV1;Q9SWF3;O03989;D7FQK6	Actin, flagellar inner arm intermediate chain	Micromonas pusilla	2	1.24	1.98E-02
A0A097PB89;D1J797;B0JWV1	ATP synthase subunit alpha	Microcystis aeruginosa	2	1.17	4.61E-03
A0A172C1L3;Q1KVS9;A0A172BZR9	Elongation factor Tu	Scenedesmus sp. CCMA_UFSCar 088	4	1.10	1.34E-02
I0YRY7;Q56D00	14-3-3 protein	Coccomyxa subellipsoidea	1	-1.13	7.37E-03
D8UHN1;E1ZLJ5	DAP decarboxylase	Volvox carteri f. nagariensis	2	-1.22	2.35E-02
A0A0C4K0H7;I0YIH9	Sedoheptulose-1,7-bisphosphatase	Coccomyxa subellipsoidea (strain C-169)	2	-1.22	2.38E-02
A8JDW2;D8U3S7	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.23	2.42E-02
D8UFR3;A8J9T0	40S ribosomal protein S12	Volvox carteri f. nagariensis	3	-1.24	1.27E-02
A0A0C4K0H7	SBP protein	Dunaliella tertiolecta	1	-1.26	1.57E-02
A8JCY4;D8U593;I0YSE8	Fructose-bisphosphate aldolase	Chlamydomonas reinhardtii	22	-1.29	2.14E-02
A8JDV2;D8UIE7	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.30	2.11E-02
A8JEU4;Q8RY44	Heat shock protein 70A	Dunaliella salina	1	-1.32	2.53E-03
I0YRY7;Q56D00;E1ZIV3	14-3-3 protein	Coccomyxa subellipsoidea	1	-1.34	3.15E-03

S4VNM6;H6X2P3;A0A110B8J4;	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.34	4.03E-06
D8U4Q1	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.36	3.62E-03
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus bijugus	2	-1.37	7.60E-04
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus sp. LU29	2	-1.38	7.59E-05
S4VNM6;H6X2P3;A0A110B8J4;A0A110B723;A0A110B8J6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.41	7.50E-05
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus sp. LU25	1	-1.43	1.09E-02
A8JEU4	Heat shock protein 70A	Chlamydomonas reinhardtii	1	-1.74	1.98E-02
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus bijugus	4	-1.88	1.08E-04
B0JM87	Uncharacterized protein	Microcystis aeruginosa	1	-1.90	1.78E-03

DW_{FLOC} vs. DW_{PLK}

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
B7TJI2	Heat shock protein 70B	Dunaliella salina	1	9.33	1.80E-02
Q1KVT0;P06541;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	2.41	6.68E-04
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus	1	2.35	4.86E-05
D8TV46	Uncharacterized protein	Volvox carteri f. nagariensis	1	2.35	2.61E-02
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4;S4VNM6;H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	1	2.28	1.23E-05
Q1KVY2	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	2.09	6.43E-03
I0YL77;E1ZL24;D8TPC8;A8IL29;A4S5Z2;C1MH11;K8FE75	ADP-ribosylation factor 1	Coccomyxa subellipsoidea	3	2.05	6.83E-03
I0YP36	Isocitrate dehydrogenase [NADP]	Coccomyxa subellipsoidea	2	1.91	1.25E-02
D7FUD3	Kinesin (Subfamily)	Ectocarpus siliculosus	1	1.82	2.34E-02
Q84X75;E1ZFR4;D8TK78	Uncharacterized protein	Chlorella variabilis	1	1.82	1.04E-02
A0A0X9AMW9;A0A0X9AGK8;S4VNM6;H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU1	1	1.80	1.39E-06
Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii	1	1.76	5.36E-03
Q1KVY2;E9NPS2;P10898;K7NU72	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	1	1.72	1.92E-03
Q1KVU8;F2YGK0	Photosystem II protein D1	Tetradesmus obliquus	1	1.57	7.32E-03
P06007;Q1KVW6;Q4JLT1;K8FE34	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	1.54	3.20E-04

A8J1G8	40S ribosomal protein S6	Chlamydomonas reinhardtii	1	1.42	2.38E-02
A8JJG8;A8JJV5;A4S1C9;K8EHQ7;C1MHL2;A8JJN6	Histone H2B	Chlamydomonas reinhardtii	3	1.42	8.52E-03
Q1KVT0;P06541;P48081;A0A097PBH6;Q8HDG4	ATP synthase subunit beta, chloroplastic	Chlamydomonas reinhardtii	1	1.41	3.85E-03
Q8HDD7	p700 chlorophyll a-apoprotein A2	Scenedesmus quadricauda	1	1.34	2.37E-02
Q1HVA2;E1ZT20;D8U9J4;A8HP84	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	Chlamydomonas reinhardtii	1	1.33	7.53E-03
D8TM08	Coproporphyrinogen III oxidase chloroplast	Volvox carteri f. nagariensis	22	1.32	5.91E-03
I0Z918	Binding protein 1	Coccomyxa subellipsoidea	1	1.30	6.29E-03
E1ZD58;I0YR87	Cysteine synthase	Chlorella variabilis	1	1.28	8.12E-03
K8ENF9	Molecular chaperone DnaK	Bathycoccus prasinos	1	1.28	6.46E-03
P06007;Q1KVW6	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	1.27	2.12E-02
D8TV46;A8IRQ1	Ribose-5-phosphate isomerase	Chlamydomonas reinhardtii	1	1.25	1.04E-02
P26526;B7U1J0;K7NRE6;A0A1C8XRI8;D0FXX3	ATP synthase subunit alpha, chloroplastic	Chlamydomonas reinhardtii	1	1.18	2.20E-02
E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea	1	-1.20	3.88E-03
E1ZBK2;D8TNN3;D8THW4;A8HX38	Eukaryotic translation initiation factor 1 alpha 1	Volvox carteri f. nagariensis	2	-1.23	1.37E-03
I0YZE5	EF-hand	Coccomyxa subellipsoidea	2	-1.25	3.90E-04
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3;I3UMQ4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	4	-1.26	1.67E-02
Q42690;D8TKY4;I0YN66;E1ZQQ5	Fructose-bisphosphate aldolase 1, chloroplastic	Chlamydomonas reinhardtii	1	-1.26	4.60E-03

D8TYV7	Phosphoglycerate kinase	Volvox carteri f. nagariensis	1	-1.48	2.56E-02
Q9FE86	2-cys peroxiredoxin, chloroplastic	Chlamydomonas reinhardtii	1	-1.48	2.42E-02
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU25	1	-1.53	7.69E-04
A0A172C1L3;Q1KVS9;A0A120N1C6	Elongation factor Tu	Scenedesmus sp. CCMA_UFSCar 088	4	-1.74	6.15E-03
S4VNM6;H6X2P3;A0A110B8J4;A0A110B723	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus obtusus	1	-1.99	1.17E-06
Q8LRU1;I0YP34;D8TX08	Ferritin	Chlamydomonas reinhardtii	1	-2.19	2.72E-03
I0YRY7;Q56D00;E1ZIV3	14-3-3 protein	Coccomyxa subellipsoidea	1	-2.21	3.62E-05
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU20	2	-2.31	9.91E-06
Q1KVS9;A0A120N1C6;A0A172BZR9	Elongation factor Tu, chloroplastic	Tetradesmus obliquus	1	-2.50	3.22E-03

DW_{FLOC} vs. ASTM

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVV6	Photosystem II CP47 reaction center protein	Tetradesmus obliquus	1	2.36	5.31E-05
Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	Chlamydomonas reinhardtii	1	1.57	2.60E-03
A8IQU3;D8TRA2;K8EN95;A4RSS5;C1MKD5;I0YLJ1	ATP synthase subunit beta	Chlamydomonas reinhardtii	1	1.50	2.82E-02
Q1KVT0;P06541;P48081;A0A097PBH6;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	1.50	4.54E-04
Q1KVT0;Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	1.48	2.03E-04
A8HXL8;E1ZEB1;D8TI16;I0ZA63	Chloroplast ATP synthase gamma chain	Chlamydomonas reinhardtii	1	1.44	1.28E-02
Q1KVY2;E9NPS2;P10898	Photosystem II CP43 reaction center protein	Coccomyxa subellipsoidea	1	1.42	9.17E-04
Q1KVU8;F2YGK0	Photosystem II protein D1	Tetradesmus obliquus	1	1.38	2.75E-03
A0A097PB89	ATP synthase subunit alpha	Cyanophora paradoxa	2	1.38	3.43E-03
E1ZBK2;D8TNN3;D8THW4;A8HX38	Uncharacterized protein	Chlorella variabilis	1	1.36	1.34E-03
Q1KVY2	Photosystem II CP43 reaction center protein	Tetradesmus obliquus	2	1.36	1.30E-02
A8HY43;D8THL7	Thylakoid lumenal protein	Chlamydomonas reinhardtii	3	1.27	1.75E-02
Q1KVU3	50S ribosomal protein L12, chloroplastic	Tetradesmus obliquus	1	1.26	8.57E-03
E1ZBK2;D8TNN3;D8THW4	Eukaryotic translation initiation factor 1 alpha 1	Volvox carteri f. nagariensis	1	1.18	4.39E-03
E1Z5I7;A8IZZ4;D8U995;E1Z8A6;D8U547	Bi-ubiquitin	Chlamydomonas reinhardtii	1	1.15	1.57E-03

D8TV46;A8IRQ1	Ribose-5-phosphate isomerase	Chlamydomonas reinhardtii	2	1.13	1.53E-02
Q1KVT0	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	1	-1.14	1.64E-04
D8UBP2	Uncharacterized protein	Volvox carteri f. nagariensis	1	-1.22	1.34E-02
A8IZU0;D8TMR1;B7TJI1;D8UI03	Heat shock protein 70C	Chlamydomonas reinhardtii	1	-1.24	1.23E-02
Q84RL9	Enolase	Dunaliella salina	1	-1.25	1.96E-03
A8J1M9;D8TL63	Thylakoid lumenal 17.4 kDa protein	Chlamydomonas reinhardtii	22	-1.25	8.32E-03
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	1	-1.28	2.24E-03
A0A0S1LH61	Peptidylprolyl isomerase	Scenedesmus sp. FKBP	4	-1.28	1.59E-03
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6;I3UMR2;I3UMQ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU29	2	-1.29	1.37E-05
D7FK90;D8LI58;D7FZN2;I0YNC4;I0YKI7;P93662	Molecular chaperones HSP70/HSC70, HSP70 superfamily	Ectocarpus siliculosus	2	-1.30	1.70E-03
A8JEU4;Q8RY44	Heat shock protein 70A	Chlamydomonas reinhardtii	1	-1.31	1.03E-03
I0YRY7	14-3-3 protein	Coccomyxa subellipsoidea	1	-1.32	2.20E-02
E9NPW9	Elongation factor Tu	Coccomyxa subellipsoidea	1	-1.32	9.78E-06
Q42690;D8TKY4;I0YN66;E1ZQQ5	Fructose-bisphosphate aldolase 1, chloroplastic	Chlamydomonas reinhardtii	2	-1.33	2.37E-07
Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	-1.37	7.59E-04
A4S0V1	Uncharacterized protein	Ostreococcus lucimarinus (strain CCE9901)	1	-1.38	1.88E-02
I0YQ64;A8J537	Catalase	Coccomyxa subellipsoidea	2	-1.40	6.13E-03

S4VNM6;H6X2P3;A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.41	9.40E-09
A0A0X9AMW9;A0A0X9AGK8;I3UMQ6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus sp. LU29	1	-1.44	3.61E-04
A0A0C4K0H7;I0YIH9	SBP protein	Dunaliella tertiolects	2	-1.46	2.16E-03
A8JCY4;D8U593;I0YSE8	Fructose-bisphosphate aldolase	Chlamydomonas reinhardtii	2	-1.53	1.32E-02
A0A0X9AMW9;A0A0X9AGK8;S4VNM6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	4	-1.78	4.81E-06

DW_{PLK} vs. ASTM

UNIPROT ID	PROTEIN NAME	ORGANISM	# UNIQUE PEPTIDES	FOLD CHANGE	P
Q1KVS9;A0A120N1C6;A0A172BZR9;A0A110B8L5	Elongation factor Tu, chloroplastic	Tetradesmus obliquus	1	3.18	8.84E-03
I0YRY7;Q56D00;E1ZIV3	14-3-3 protein	Coccomyxa subellipsoidea	1	3.06	2.63E-05
Q8LRU1;I0YP34;D8TX08	Ferritin	Chlamydomonas reinhardtii	1	2.58	6.29E-03
A0A0X9AMW9	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	1	2.12	5.08E-05
S4VNM6;H6X2P3;A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus armatus	1	2.00	1.18E-05
D8TTX1	Uncharacterized protein	Volvox carteri f. nagariensis	1	1.98	6.64E-03
Q1KVT0;Q8HDG4	ATP synthase subunit beta, chloroplasti	Tetradesmus obliquus	1	1.90	9.79E-05
D8UI03;A8HYV3	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.88	2.04E-02
A0A172C1L3;Q1KVS9;A0A120N1C6	Elongation factor Tu	Scenedesmus sp. CCMA_UFSCar 088	1	1.86	3.94E-03
D8UFR3;A8J9T0	40S ribosomal protein S12	Volvox carteri f. nagariensis	3	1.80	1.72E-03
E1ZBK2;D8TNN3;D8THW4;A8HX38	Eukaryotic translation elongation factor 1 alpha 2	Volvox carteri f. nagariensis	3	1.67	2.92E-04
D8UI03;A8HYV3;E1ZE03	HSP70bf (Heat shock protein 70B)	Volvox carteri f. nagariensis	1	1.58	2.41E-02
Q1KVU3	50S ribosomal protein L12, chloroplastic	Tetradesmus obliquus	2	1.40	1.27E-02
I0YZE5	EF-hand	Coccomyxa subellipsoidea	1	1.36	3.44E-05

E1Z5I7;A8IZZ4;D8U995;E1Z8A6	Bi-ubiquitin	Chlamydomonas reinhardtii	1	1.35	1.10E-03
E1ZBK2;D8TNN3;D8THW4	Eukaryotic translation initiation factor 1 alpha 1	Volvox carteri f. nagariensis	1	1.29	1.16E-04
K8F4N5	3-oxoacyl-[acyl-carrier-protein] synthase	Bathycoccus prasinos	1	1.22	2.80E-02
Q1KVT0	ATP synthase subunit beta, chloroplasti	Tetradesmus obliquus	2	-1.12	4.28E-03
S4VNM6;H6X2P3;A0A110B8J4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large Subunit	Scenedesmus armatus	1	-1.21	6.38E-03
Q1KVY1	ATP synthase subunit b, chloroplasti	Tetradesmus obliquus	1	-1.21	2.71E-02
I0Z918	Binding protein 1	Coccomyxa subellipsoidea	1	-1.23	2.67E-03
A0A1B0VE51	Superoxide dismutase	Scenedesmus acutus	1	-1.25	1.16E-02
I0YQ64;A8J537	Catalase	Chlamydomonas reinhardtii	1	-1.31	1.66E-02
Q8HDG4	ATP synthase subunit beta	Scenedesmus quadricauda	1	-1.33	2.60E-04
P06007;Q1KVW6	Photosystem II D2 protein	Chlamydomonas reinhardtii	1	-1.38	1.17E-02
D8TM08	Coproporphyrinogen III oxidase chloroplast	Volvox carteri f. nagariensis	1	-1.41	2.20E-02
Q84X75;E1ZFR4;D8TK78	CR051 protein (Predicted protein)	Chlamydomonas reinhardtii	1	-1.52	2.14E-02
I0YL77;E1ZL24;D8TPC8	ADP-ribosylation factor 1	Coccomyxa subellipsoidea	1	-1.81	3.43E-03
Q1KVT0;P06541;Q8HDG4	ATP synthase subunit beta, chloroplastic	Tetradesmus obliquus	3	-2.38	1.01E-03
A0A0X9AMW9;A0A0X9AGK8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus bijugus	4	-2.95	2.12E-06
D8TV46	Uncharacterized protein	Volvox carteri f. nagariensis	1	-3.12	2.77E-02
A0A0X9AMW9;A0A0X9AGK8;S4VNM6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Scenedesmus armatus	1	-3.22	3.10E-07

SECTION III – VENN DIAGRAMS

UNIQUE DEPS RELATIVE TO PHENOTYPES COMPARISONS AMONG EXPERIMENTAL GROUPS- ITRAQ#1

PHENOTYPE COMPARISON. UNIQUE DEPS	UNIPROT ID	PROTEIN NAME	REGULATION
DWFLOC VS CONTROL	Q1KVY3	Photosystem I P700 chlorophyll a apoprotein A1	up
	I0YS06	GTP-binding protein YPTC1	up
	D8UI88	Uncharacterized protein (catalytic activity)	up
	E1ZQY4	40S ribosomal protein S5	up
	I0YUW3	Elongation factor 2	up
	Q9FEK6	Chlorophyll a-b binding protein, chloroplastic	up
	E1ZD58	Cysteine synthase	up
	D8TZZ8	Uncharacterized protein (protein folding)	up
	A8IWQ7	Predicted protein (protein repair; response to oxidative stress)	up
	A8J5P7	Ubiquinol:cytochrome c oxidoreductase 50 kDa core 1 subunit	down
	H6X2F8	Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit	down
	D8UBP2	Uncharacterized protein (Rhodanese-like domain)	down
	D8TUP1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	down
	I0Z401	Ferredoxin-dependent glutamate synthase	down
	G4WUV9	Chloroplast ATP synthase gamma chain protein	down

	K8F9G7	Uncharacterized protein (Bromodomain)	down
	H6X2P3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	down
DWPLK VS CONTROL	Q1KVU8	Photosystem II protein D1	up
	A8JJG8	Histone H2B	up
	Q1KVY2	Photosystem II CP43 reaction center protein	up
	D8U3K8	Uncharacterized protein (photosynthesis)	up
	A8J1G8	40S ribosomal protein S6	up
	I0YP36	Isocitrate dehydrogenase [NADP]	up
	E1Z6L2	Uncharacterized protein (carbohydrate metabolic process)	down
	D8U1T0	Uncharacterized protein	down
ASTM VS CONTROL	E1ZBK2	Uncharacterized protein (GTPase activity; GTP binding)	up
	I0YQ64	Catalase	up
	I0Z028	Vitamin B6 biosynthesis protein	up
	A4S0V1	Uncharacterized protein (L-malate dehydrogenase activity; carbohydrate metabolic process; tricarboxylic acid cycle)	up
	D8UI03	HSP70bf (Heat shock protein 70B)	up
	A0A0S1LH61	Peptidylprolyl isomerase	up
	D8TV46	Uncharacterized protein (pentose-phosphate shunt, non-oxidative branch)	up
	E9NPW9	Elongation factor Tu	up
	D8U1R3	Uncharacterized protein (protein metabolic process)	up

	A8IQU3	ATP synthase subunit beta	up
	A0A0C4K0H7	SBP protein	up
	D8UFR3	40S ribosomal protein S12	up
	D7FK90	Molecular chaperones HSP70/HSC70, HSP70 superfamily	up
	I0YRY7	14-3-3 protein	up
	Q1KVS9	Elongation factor Tu, chloroplastic	up
	A0A172C1L3	Elongation factor Tu	up
	B0JXA3	Phycocyanin beta subunit	up
	A8JBG5	Flavoprotein	up
	A8IZU0	Heat shock protein 70C	up
	A8IX80	Acetohydroxyacid dehydratase	down
	Q42690	Fructose-bisphosphate aldolase 1, chloroplasti	down
	A8IW00	Glutamine synthetase	down
	D8TK12	Glucose-6-phosphate isomerase	down
	Q8HDG4	ATP synthase subunit beta	down
	I0YZ27	Glyoxalase I	down
	A4RTP0	Malate dehydrogenase	down
	Q84RL9	Enolase	down
	C1MNA2	Predicted protein (proteolysis)	down

	D8TZD7	Uncharacterized protein (protein refolding)	down
	Q8VXQ9	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	down
DW FLOC VS. DW PLK	P06541	ATP synthase subunit beta, chloroplastic	Up
	D8U1T0	Uncharacterized protein	Up
	A8HYU5	S-adenosylmethionine synthase	Up
	A8JJG8	Histone H2B	Down
	D8UF20	Uncharacterized protein	Down
	A8IYP4	Phosphoribulokinase	Down
DW FLOC VS. ASTM	A8IQU3	ATP synthase subunit beta	Up
	I0YUW3	Elongation factor 2	Up
	I0YP36	Isocitrate dehydrogenase [NADP]	Up
	D8TRA2	ATP synthase subunit beta	Up
	D8TNN3	Eukaryotic translation elongation factor 1 alpha 2	Up
	A8ISB0	Cysteine synthase	Up
	A0A097PB89	ATP synthase subunit alpha	Up
	Q84RL9	Enolase	Down
	A8IZU0	Heat shock protein 70C	Down
	I0YQ64	Catalase	Down

	E9NPW9	Elongation factor Tu	Down
	I0Z401	Ferredoxin-dependent glutamate synthase	Down
	A4S0V1	Uncharacterized protein (carbohydrate metabolic process; tricarboxylic acid cycle)	Down
	Q42690	Fructose-bisphosphate aldolase 1, chloroplastic	Down
	A8IW00	Glutamine synthetase	Down
	D7FK90	Molecular chaperones HSP70/HSC70, HSP70 superfamily	Down
	Q1KVY2	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	Down
	Q8HDG4	ATP synthase subunit beta	Down
	K8F9G7	Uncharacterized protein (Bromodomain)	Down
	A8JCY4	Fructose-bisphosphate aldolase	Down
	D8TUP1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Down
	Q1KVT2	Cytochrome b6	Up
DW PLK VS. ASTM	A4SB22	Uncharacterized protein (GTPase activity; GTP binding)	Up
	D8TV46	Uncharacterized protein (pentose-phosphate shunt, non-oxidative branch)	Down

UNIQUE DEPS RELATIVE TO PHENOTYPES COMPARISONS AMONG EXPERIMENTAL GROUPS- ITRAQ#2

PHENOTYPE COMPARISON	UNIPROT ID	PROTEIN NAME	REGULATION
DW FLOC VS. CONTROL	P37255	Photosystem II CP47 reaction center protein	up
	A8HXL8	Chloroplast ATP synthase gamma chain	up
	I0Z5X3	Uncharacterized protein (electron carrier activity; heme binding; metal ion binding)	up
	P10898	Photosystem II CP43 reaction center protein	up
	A8JJV5	Histone H2B	up
	A8J1G8	40S ribosomal protein S6	up
	C1MYV3	Actin, flagellar inner arm intermediate chain	up
	D8UHN1	DAP decarboxylase	down
	A8JDW2	Predicted protein	down
	A8JCY4	Fructose-bisphosphate aldolase	down
	I3UMQ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	down
	I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	down
DW PLK VS. CONTROL	Q8LRU1	Ferritin	up
	Q1KVS9	Elongation factor Tu, chloroplastic	up
	D8UI03	HSP70bf (Heat shock protein 70B)	up

	E1ZQL8	Glutamate-1-semialdehyde 2,1-aminomutase	up
	E1Z5I7	Uncharacterized protein (Ubiquitinin like domain)	up
	E1ZMW8	Uncharacterized protein (phosphatase activity)	up
	I0YZE5	EF-hand	up
	A8J6C7	Membrane AAA-metalloprotease	up
	E1Z6L2	Uncharacterized protein (carbohydrate metabolic process)	down
	Q84X75	CR051 protein (Predicted protein)	down
	D8UBQ8	ADP-glucose pyrophosphorylase	down
	I0YP36	Isocitrate dehydrogenase [NADP]	down
	C1MXS6	Predicted protein	down
	B7TJI2	Heat shock protein 70B	down
	Q8HDG4	ATP synthase subunit beta	up
	D8TV46	Uncharacterized protein (pentose-phosphate shunt, non-oxidative branch)	up
	A4S0V1	Uncharacterized protein (carbohydrate metabolic process; tricarboxylic acid cycle)	up
ASTM VS. CONTROL	A8IZU0	Heat shock protein 70C	up
	A0A0S1LH61	Peptidylprolyl isomerase	up
	A4RTP0	Malate dehydrogenase	up
	Q42690	Fructose-bisphosphate aldolase 1, chloroplasti	up

	A8IW00	Glutamine synthetase	up
	P06541	ATP synthase subunit beta, chloroplastic	up
	Q84RL9	Enolase	up
	A0A1B0VE51	Superoxide dismutase	up
	D7FK90	Molecular chaperones HSP70/HSC70, HSP70 superfamily	up
	D8U477	Uncharacterized protein (terpenoid biosynthetic process)	up
	D8U5B1	Large subunit of isopropylmalate dehydratase	down
	I0YUW3	Elongation factor 2	down
	K8F4N5	3-oxoacyl-[acyl-carrier-protein] synthase	down
	I0Z401	Ferredoxin-dependent glutamate synthase	down
	E1ZBK2	Uncharacterized protein (GTPase activity; GTP binding)	down
	A4RQS5	Phosphomannomutase	down
	P48101	Magnesium-chelatase subunit ChlI	down
	D8TK12	Glucose-6-phosphate isomerase	down
	D8TNN3	Eukaryotic translation elongation factor 1 alpha 2	down
	D8TJY9	Sedoheptulose-1,7-biphosphatase	down
	I0YP36	Isocitrate dehydrogenase [NADP]	up
DW FLOC VS. DW PLK	D7FUD3	Kinesin (Subfamily)	up

DW FLOC VS. ASTM	A8J1G8	40S ribosomal protein S6	up
	A8JJV5	Histone H2B	up
	Q8HDD7	p700 chlorophyll a-apoprotein A2	up
	Q1HVA2	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	up
	E1ZD58	Cysteine synthase	up
	K8ENF9	Molecular chaperone DnaK	up
	Q1KVW6	Photosystem II D2 protein	up
	P26526	ATP synthase subunit alpha, chloroplastic	up
	D8TYV7	Phosphoglycerate kinase	down
	Q9FE86	Thioredoxin peroxidase	down
	I3UMR2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	down
	I3UMQ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	down
	A8IQU3	ATP synthase subunit beta	up
	A8HXL8	Chloroplast ATP synthase gamma chain	up
	A0A097PB89	ATP synthase subunit alpha	up
	A8HY43	Thylakoid lumenal protein	up
	D8TNN3	Eukaryotic translation elongation factor 1 alpha 2	up
	D8UBP2	Uncharacterized protein (Rhodanese-like domain)	down

DW PLK VS. ASTM	A8IZU0	Heat shock protein 70C	down
	Q84RL9	Enolase	down
	A8J1M9	Thylakoid lumenal 17.4 kDa protein	down
	A0A0S1LH61	Peptidylprolyl isomerase	down
	D7FK90	Molecular chaperones HSP70/HSC70, HSP70 superfamily	down
	A8JEU4	Heat shock protein 70A	down
	A4S0V1	Uncharacterized protein	down
	A8JCY4	Fructose-bisphosphate aldolase	down
	D8TTX1	Uncharacterized protein	up
	D8UI03	HSP70bf (Heat shock protein 70B)	up
	D8UFR3	40S ribosomal protein S12	up
	K8F4N5	3-oxoacyl-[acyl-carrier-protein] synthase	up
	Q1KVY1	ATP synthase subunit b, chloroplastic	down
	A0A1B0VE51	Superoxide dismutase	down

SECTION IV - BRITE FUNCTIONAL HIERARCHIES

Time Point	*Phenotype Comparisons*	*UniProt ID*	*BriteHierarchy*		
2h	DW floc vs Control	Q1KVY3	Metabolism	Energy Metabolism	Photosynthesis
		Q9FEK6	Metabolism	Energy Metabolism	Photosynthesis
		E1ZD58	Metabolism	Energy Metabolism	Sulfur metabolism
		A8J5P7	Metabolism	Enzyme Families	Peptidases
		H6X2F8	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
		D8TUP1	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
		I0Z401	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
		G4WUV9	Metabolism	Energy Metabolism	Oxidative Phosphorylation
		I0YS06	Genetic Information Processing	Folding, sorting and degradation	Membrane Trafficking
		E1ZQY4	Genetic Information Processing	Translation	Ribosome
		K8F9G7	Genetic Information Processing	Transcription	Basal Transcription Factors
		I0YUW3	Environmental Information Processing	Signal transduction	AMPKsignalingpathway
	DW plk vs Control	Q1KVU8	Metabolism	Energy Metabolism	Photosynthesis
		Q1KVY2	Metabolism	Energy Metabolism	Photosynthesis
		D8U3K8	Metabolism	Energy Metabolism	Photosynthesis

ASTM vs Control	I0YP36	Metabolism	Carbohydrate Metabolism	Citrate Cycle (TCA cycle)
	E1Z6L2	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	A8JJG8	Cellular Processes	Transport and Catabolism	Exosome
	D8U1T0	Spliceosome	Other splicing related proteins	Spliceosome associated proteins (SAPs)
	A8J1G8	Environmental Information Processing	Signal transduction	Apelin signaling pathway
	I0YQ64	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
	I0Z028	Metabolism	Metabolism of cofactors and vitamins	VitaminB6metabolism
	A4S0V1	Metabolism	Carbohydrate Metabolism	Citrate Cycle (TCA cycle)
	D8TV46	Metabolism	Carbohydrate Metabolism	Pentose Phosphate Pathway
	A0A0C4K0H7	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	D7FK90	Metabolism	Enzyme Families	Protein Phosphatase and Associated Proteins
	A8IX80	Metabolism	Aminoacidmetabolism	Valine, leucine and isoleucine biosynthesis
	Q42690	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	A8IW00	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
	D8TK12	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	Q8HDG4	Metabolism	Energy Metabolism	Oxidative Phosphorylation

DW floc vs ASTM	A4RTP0	Metabolism	Carbohydrate Metabolism	Citrate Cycle (TCA cycle)
	Q84RL9	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	C1MNA2	Metabolism	Enzyme Families	Peptidases
	Q8VXQ9	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	E1ZBK2	Genetic Information Processing	Translation	RNA Transport
	D8U1R3	Genetic Information Processing	Folding, sorting and degradation	Chaperones and Folding Catalysts
	A8IQU3	Genetic Information Processing	Translation	Mitochondrial biogenesis
	D8UFR3	Genetic Information Processing	Translation	Ribosome
	I0YRY7	Genetic Information Processing	Replicationandrepair	DNA repair and recombination proteins
	A0A0S1LH61	Genetic Information Processing	Folding, sorting and degradation	Chaperones and folding catalysts
	A8IZU0	Genetic Information Processing	Folding, sorting and degradation	RNA degradation
	D8TZD7	Genetic Information Processing	Folding, sorting and degradation	RNA degradation
	D8UI03	Environmental Information Processing	Signal transduction	MAPK signalingpathway
	E9NPW9	Cellular Processes	Transport and Catabolism	Exosome
	Q1KVS9	Cellular Processes	Transport and Catabolism	Exosome
	A8IQU3	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	I0YP36	Metabolism	Carbohydrate Metabolism	Citrate Cycle (TCA cycle)

	D8TRA2	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	A8ISB0	Metabolism	Energy Metabolism	Sulfurmetabolism
	A0A097PB89	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	Q84RL9	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	I0YQ64	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
	I0Z401	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
	A4S0V1	Metabolism	Carbohydrate Metabolism	Citrate Cycle (TCA cycle)
	Q42690	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	A8IW00	Metabolism	Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism
	Q1KVY2	Metabolism	Energy Metabolism	Photosynthesis
	Q8HDG4	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	A8JCY4	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	D8TUP1	Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis
	D8TNN3	Genetic Information Processing	Translation	RNAtransport
	A8IZU0	Genetic Information Processing	Folding, sorting and degradation	RNAdegradation
	K8F9G7	Genetic Information Processing	Transcription	Basal Transcription Factors
	E9NPW9	Cellular Processes	Transport and Catabolism	Exosome
	I0YUW3	Environmental Information	Signal transduction	AMPK signaling pathway

			Processing		
		D7FK90	Environmental Information Processing	Signal transduction	MAPK signaling pathway
	DWplk vs ASTM	Q1KVT2	Metabolism	Energy Metabolism	Photosynthesis
		D8TV46	Metabolism	Carbohydrate Metabolism	Pentose Phosphate Pathway
		A4SB22	Environmental Information Processing	Signal transduction	MAPK signaling pathway
20h	DW floc vs Control	P37255	Metabolism	Energy metabolism	Photosynthesis
		A8HXL8	Metabolism	Energy Metabolism	Oxidative Phosphorylation
		I0Z5X3	Metabolism	Energy Metabolism	Oxidative Phosphorylation
		P10898	Metabolism	Energy Metabolism	Photosynthesis
		D8UHN1	Metabolism	Aminoacidmetabolism	Lysine Biosynthesis
		A8JCY4	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
		I3UMQ3	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
		I3UMR2	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
		A8JJV5	CellularProcesses	Transportandcatabolism	Exosome
		C1MYV3	CellularProcesses	Cellmobility	Cytoskeleton Proteins
		A8J1G8	Environmental Information Processing	Signal Transduction	Apelin signaling pathway
	DW plk vs Control	E1ZQL8	Metabolism	Metabolismofcofactorsandvitamins	Porphyrin and Chlorophyll Metabolism

	A8J6C7	Metabolism	Enzymefamilies	Peptidases
	E1Z6L2	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	Q84X75	Metabolism	Lipids Metabolism	Fatty Acids Biosynthesis
	D8UBQ8	Metabolism	Carbohydrates Metabolism	Starch and Sucrose Metabolism
	I0YP36	Metabolism	Carbohydrates Metabolism	Citrate Cycle (TCA cycle)
	E1ZMW8	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
	Q8LRU1	CellularProcesses	Cell Growth and Death	Ferroptosis
	Q1KVS9	CellularProcesses	Transportandcatabolism	Exosome
	E1Z5I7	Genetic Information Processing	Folding, sorting and degradation	Ubiquitin system
	C1MXS6	Genetic Information Processing	Translation	Mitochondrial biogenesis
	D8UI03	Environmental Information Processing	Signal Transduction	MAPK signaling pathway
	I0YZE5	Environmental Information Processing	Signal Transduction	MAPK signaling pathway
ASTM vs Control	Q8HDG4	Metabolism	Carbohydrates Metabolism	Pentose Phosphate Pathway
	D8TV46	Metabolism	Carbohydrates Metabolism	Citrate Cycle (TCA cycle)
	A0A0S1LH61	Metabolism	Carbohydrates Metabolism	Citrate Cycle (TCA cycle)
	A4RTP0	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis

	Q42690	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
	A8IW00	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	P06541	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
	A0A1B0VE51	Metabolism	Enzymefamilies	Protein phosphatase and associated proteins
	D7FK90	Metabolism	Metabolismofterpenoidsandpolyketides	Terpenoid backbone biosynthesis
	D8U477	Metabolism	Carbohydrates Metabolism	C5-Branched dibasic acid metabolism
	I0YUW3	Metabolism	Lipids Metabolism	Fatty Acids Biosynthesis
	K8F4N5	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
	E1ZBK2	Metabolism	Carbohydrates Metabolism	Fructoseandmannosemetabolism
	A4RQS5	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
	D8TK12	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	A4S0V1	Genetic Information Processing	Folding, sorting and degradation	RNA degradation
	A8IZU0	Genetic Information Processing	Folding, sorting and degradation	Chaperones and folding catalysts
	I0Z401	Genetic Information Processing	Translation	RNAtransport
	P48101	Genetic Information Processing	Translation	RNAtransport
	Q84RL9	Environmental Information Processing	Signal Transduction	SOD2; superoxidedismutase, Fe-Mnfamily

	D8U5B1	Environmental Information Processing	Signal Transduction	AMPK signaling pathway
DW floc vs DW plk	I0YP36	Metabolism	Carbohydrates Metabolism	Citrate Cycle (TCA cycle)
	Q8HDD7	Metabolism	Energy Metabolism	Photosynthesis
	Q1HVA2	Metabolism	Energy Metabolism	Carbon Fixation in Photosynthetic Organisms
	E1ZD58	Metabolism	Energy Metabolism	Sulfurmetabolism
	Q1KVW6	Metabolism	Energy Metabolism	Photosynthesis
	P26526	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	D8TYV7	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
	I3UMR2	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
	I3UMQ3	Metabolism	Carbohydrates Metabolism	Glyoxylate and dicarboxylate metabolism
	D7FUD3	CellularProcesses	Cell motility	Cytoskeleton Proteins
	A8JJV5	CellularProcesses	Transportandcatabolism	Exosome
	Q9FE86	CellularProcesses	Transportandcatabolism	Exosome
	A8J1G8	Environmental Information Processing	Signal Transduction	Apelin signaling pathway
	K8ENF9	Environmental Information Processing	Signal Transduction	MAPK signaling pathway
DW floc vs ASTM	A8HXL8	Metabolism	Energy Metabolism	Oxidative Phosphorylation

DW plk vs ASTM	A0A097PB89	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	D8UBP2	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
	A8J1M9	Metabolism	Carbohydrates Metabolism	Citrate Cycle (TCA cycle)
	A0A0S1LH61	Metabolism	Carbohydrates Metabolism	Glycolysis/Gluconeogenesis
	A8IQU3	Genetic Information Processing	Translation	Mitochondrialbiogenesis
	A8HY43	Genetic Information Processing	Translation	RNAtransport
	D8TNN3	Genetic Information Processing	Folding, sorting and degradation	RNAdegradation
	A8IZU0	Genetic Information Processing	Folding, sorting and degradation	Chaperonesandfoldingcatalysts
	Q84RL9	Environmental Information Processing	Signal Transduction	MAPK signaling pathway
	D8UI03	Metabolism	Enzymefamilies	Proteinphosphataseandassociatedproteins
	K8F4N5	Metabolism	Lipids Metabolism	Fatty Acids Biosynthesis
	Q1KVY1	Metabolism	Energy Metabolism	Oxidative Phosphorylation
	D8UFR3	Genetic Information Processing	Translation	Ribosome
	A0A1B0VE51	Environmental Information Processing	Signal Transduction	SOD2; superoxidedismutase, Fe-Mnfamily

CHAPTER VI

Thesis Conclusions & Future Directions

6.1 Introduction

Microalgae are miniature cell factories that can be cultivated for a variety of products such as pigments, nutraceuticals, cosmetics, animal feed and fertilizers. However, process improvements are required on a large scale, including reduction of harvesting costs (Uduman et al., 2010) whilst miminising biomass and medium contamination (Vandamme et al., 2013); to this regard, a combined ecology-engineering approach may provide an effective solution. Flocculation – inducing algae clumping - is considered one of the most promising economic approaches for pre-concentrating very large amounts of algal biomass, ultimately facilitating sustainable cell harvesting and reducing processing costs (Barros et al., 2015). Harvesting of biomass requires a 'clumping agent'; metal salts like ferric chloride, while effective and commonly used, are required in high dosages and contaminate both product and water medium. Polymers like chitosan are also used, representing a safer but more expensive alternative to metal salts (Vandamme et al., 2013).

In the present work, a bio-flocculation system to harvest microalgae biomass was investigated. In contrast to efforts examining auto-flocculating algae or bacterial products, the focus here was on the ecological phenomenon of predator induced bio-flocculation. Chemical cues released by grazers like Daphnia and known as infochemicals can induce colony formation and other morphological changes in several microalgae species. The induced formation of colonies and flocs/aggregates in algae has long fascinated ecologists and evolutionary biologists (Hessen and van Donk, 1993, Lürling, 2003, Pohnert et al., 2007, Fischer et al., 2014). Only recently have these induced responses been seen as a potential option for clean, low cost harvesting

of microalgae, for low - medium value products (Montemazzani et al., 2015, Zhu et al., 2017).

This thesis aimed to investigate the phenomenon from a biotechnology perspective, to better understand the biological process, and evaluate the potential for its exploitation within industry. The focus was on *Scenedesmus subspicatus*, and the zooplanktonic grazer *Daphnia magna.* Several aspects of grazer-induced flocculation, drawing on ecology, physiology and proteomics were explored. The main objectives were to assess 1) whether grazer cues were effective at inducing flocs and to what extent, 2) distinguish between colony and floc formation from a physiological and biochemical perspective, and 3) reveal cellular mechanisms that might be driving these responses, and reveal features that could either be exploited using synthetic biology approaches or with process engineering solutions.

6.2 Key Findings & Future Perspectives

Controlled flocculation of microalgae through infochemicals is a promising technology; therefore, several specific issues related to their application were addressed.

Infochemicals are likely to be species/strain specific, therefore it was important to investigate any specificity as this could impact on strain selection for industrial biomanufacturing. Also, the effect size of grazer cues was not estimated yet to allow a standardized comparison among various *Daphnia* grazers. The meta-analysis shown in Chapter II facilitated investigations into these mechanisms by synthesizing several metrics of colony size, such as cell number and overall colony size, and providing a quantitative assessment of the importance of microalgae-grazers species-specific interactions (Roccuzzo et al., 2016). From these results, it emerged which future work would need to be undertaken from both an engineering and biology perspective, such

as 1) the design of integrated methods able to provide infochemical-rich water for harvesting algal biomass and centered on recirculation of *Daphnia* cues medium in the cultivation pond and 2) characterization of the infochemicals (via mass spectrometry, for example), and the molecular processes behind the induced responses.

Chapter III provided an experimental investigation of key parameters associated with flocculation (i.e. initial algal concentration and culture age, infochemicals dosage, flocs PSD and cell surface properties) and allowing a feasible and efficient bio-flocculation approach. Possible mechanisms of actions were also investigated, and the processes of colony formation and flocculation were clearly distinguished, so providing a better understanding of the cellular responses mainly contributing to flocculation. Results showed how best flocculation performances were achieved at early exponential stage; also, at any stage of algal growth, there was no evidence of charge neutralization-like or bridging mechanisms but rather a biochemical stimulus; hence, it was hypothesised that the flocculation process was rather driven by the production of EPS, either in higher amount or with different distribution of components (Chapter IV). Therefore, the focus was on the analysis of sEPS of *S. subpsicatus*, specifically in terms of abundance of carbohydrates, proteins and uronic acids. While microscopy images seemed to indicate the presence of EPS surrounding cells and accumulating in the inner part of the algal flocs, surprisingly, no significant difference in the amounts of any of the sEPS components under study was found between exposed and non-exposed algae. The only exception was represented by the "other" fraction, speculated as composed by "small molecules, remnants of lipid based materials". These results suggested that sEPS production could account for inducing flocculation in *S. subspicatus*; however, further investigations would be necessary. Areas of

interest were identified in 1) quantification of lipids, lipo-polysaccharides or lipo-proteins in the sEPS (via LC-MS for example), 2) evaluation of different and/or fine-tuned extraction protocols, 3) full characterisation of the individual sEPS components via more advanced techniques (i.e. HPLC, mass spectrometry) and 4) use of more advanced staining or microscopy techniques for the analyisis of the flocs, such as SEM and TEM (scan/transmission electron microscopy).

Pathways and functions linked to EPS production, flocculation and colony formation in microalgae and cyanobacteria could also be analysed with omics approaches (Prochnik et al., 2010, Gulez et al., 2014, Schmid et al., 2015, Yu et al., 2015, Khona et al., 2016, Harke et al., 2017). Therefore, the focus of Chapter V was on the proteomic response of *S. subspicatus* to naturally occurring infochemicals from the grazer *D. magna*. This was the first study unravelling the molecular mechanisms behind the flocculation of the microalga *S. subpicatus* in response to *D. magna* cues. Results indicated this infochemicals induced bio-flocculation occur at the alarm phase and requires increased energy resources; also, an important role was envisaged in the synthesis of cysteine, a primary amino acid, precursors of defense biomolecules and promoter of bio-flocculation through the production of structural stable extra-cellular proteins with disulphide bonds (Xie et al., 2013, Romero et al., 2014, Aziz et al., 2016, Shi et al., 2017). Higher abundance of proteins related to photosynthesis, coupled with decreased protein abundance for carbohydrates metabolism, suggested bio-flocculation is promoted by production of different molecules other than polysaccharides and which would constitute the EPS matrix responsible for holding algal cells together. The data also indicated infochemicals induced flocculation may be sustained through MAPK signalling cascades. Conversely to flocculation, colony

formation required higher energy demands at the alarm phase which later decreased at the acclimation stage, therefore suggesting a trade-off between colony formation and support of floc form. Finally, results suggested a role of fatty acids metabolism in the process of colony formation, as they contribute to the several cellular functions, including the accurate separation of membranes during cell division. Nevertheless, further investigations would be needed and future research should focus on 1) matching the existing mass spectra to an up to date, annotated proteome database for this specific microalgal species to improve the number of proteins quantified, 2) evaluating the membrane proteome of *S. subspicatus* in response to *Daphnia* cues and its their role in algal cells adhesion and other cellular functions (i.e. molecular transport, signal transduction) and 3) identifying the key components in MAPK signalling pathways and regulating infochemicals induced bioflocculation via for example a phosphoproteomic approach.

The experimental evaluations reported in this thesis can be particularly valuable to the manufacturing industry of low-medium value algal products, where flocculation is a key step to achieve economical and sustainable biomass harvesting. Several techniques were applied to better undertand *Daphnia* induced flocculation of *S. subpsicatus*, together with an interpretation of the findings from the perspective of incorporating it into engineering practice. Future engineering work could be directed towards the application of these algal induced defense responses with the key parameters provided into mesocosm experiments and further scale-up. One option could be represented by the direct addition of refined infochemicals; their production and purification however would represent an additional cost. This could be decreased considering instead an infochemicals production system fully integrated in the algal

cultivation unit. In this case, simple modifications in the cultivation vessel design would accomplish the goal, as for example the filtration of the grazers followed by the recirculation of the infochemicals-rich medium or the addition of a "*Daphnia* pool" equipped with permeable membranes for the controlled release of the cues. Infochemicals induced bio-flocculation proved to be a relatively slow process and with better performance with algae at early exponential stage. However, from a biotechnology perspective it would be desirable to have a faster flocculation process and with algae at a later growth stage, characterised by a higher biomass density. This could be achieved by fine-tuning the infochemicals production procedure, i.e. increasing the concentration of animals or optimizing the amount of cues per algal cell. To this regard, potential is also envisaged in the field of synthetic biology, especially in the re-design of this natural biological system to embed it with predictable functions allowing to control the timing and efficiency of flocculation.

REFERENCES – CHAPTER VI

1. Aziz M, Nadipalli RK, Xie X, Sun Y, Surowiec K, Zhang JL, Paré PW. Augmenting sulfur metabolism and herbivore defense in Arabidopsis by bacterial volatile signaling. Frontiers in plant science. 2016 Apr 8; 7:458.

2. Gulez G, Altintas A, Fazli M, Dechesne A, Workman CT, Tolker-Nielsen T, et al. Colony morphology and transcriptome profiling of Pseudomonas putida KT2440 and its mutants deficient in alginate or all EPS synthesis under controlled matric potentials. Microbiologyopen. 2014;3(4):457-69.

3. Harke MJ, Jankowiak JG, Morrell BK, Gobler CJ. Transcriptomic Responses in the Bloom-Forming Cyanobacterium Microcystis Induced during Exposure to Zooplankton. Applied and Environmental Microbiology. 2017;85(4): e02832-16.

4. Khona DK, Shirolikar SM, Gawde KK, Hom E, Deodhar MA, D'Souza JS. Characterization of salt stress-induced palmelloids in the green alga, Chlamydomonas reinhardtii. Algal Research-Biomass Biofuels and Bioproducts. 2016; 16:434-48.

5. Prochnik SE, Umen J, Nedelcu AM, Hallmann A, Miller SM, Nishii I, et al. Genomic Analysis of Organismal Complexity in the Multicellular Green Alga Volvox carteri. Science. 2010;329(5988):223-6.

6. Roccuzzo S, Beckerman AP, Pandhal J. The use of natural infochemicals for sustainable and efficient harvesting of the microalgae Scenedesmus spp. for biotechnology: insights from a meta-analysis. Biotechnology Letters. 2016;38(12):1983-90.

7. Romero LC, Angeles Aroca M, Laureano-Marin AM, Moreno I, Garcia I, Gotor C. Cysteine and Cysteine-Related Signaling Pathways in Arabidopsis thaliana. Molecular Plant. 2014;7(2):264-76.

8. Schmid J, Sieber V, Rehm B. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Frontiers in Microbiology. 2015;6: 496

9. Shi J, Chen Y, Xu Y, Ji D, Chen C, Xie C. Differential Proteomic Analysis by iTRAQ Reveals the Mechanism of Pyropia haitanensis Responding to High Temperature Stress. Scientific Reports. 2017;7.

10. Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy. 2010;2(1):012701.

11. Vandamme D, Foubert I, Muylaert K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology. 2013;31(4):233-9.

12. Xie GJ, Liu BF, Xing DF, Nan J, Ding J, Ren NQ. Photo-fermentative bacteria aggregation triggered by L-cysteine during hydrogen production. Biotechnology for biofuels. 2013 Dec;6(1):64.

13. Yu W, Chen Z, Shen L, Wang Y, Li Q, Yan S, et al. Proteomic profiling of Bacillus licheniformis reveals a stress response mechanism in the synthesis of extracellular polymeric flocculants. Biotechnology and Bioengineering. 2016;113(4):797-806.