Optimising cell wall disruption of microalgae biomass for release of nutrients and bioactive compounds for aquafeed and food applications

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Abstract

Microalgae are photosynthetic organisms and primary producers of essential nutrients in the aquatic food chain. Although currently less exploited, microalgae culture can provide nutrients to cover the increasing demand for sustainable food production. Most microalgae possess hard cellulosic cell walls and cell wall integrity may significantly limit nutrient bioavailability for instance in farmed animals, such as fish, or in humans. Thus, preprocessing to disrupt the cell wall is necessary to facilitate nutrient release. The present study aimed to optimize cell wall disruption using bead milling for release of nutrients, such as lipids and proteins, and bioactive compounds, such as carotenoids and phenolic compounds, in *Tetraselmis chuii* and *Phaeodactylum tricornutum*. Bead type, level of algae dry matter, flow rate, tip speed, bead filling, and chamber volume were the tested bead milling parameters. Cell wall disruption efficiency was investigated for correlation with dry matter content, flow rate (as a measure of the retention time of the biomass in the milling chamber), and mill agitator tip speed. For *Tetraselmis chuii,* bead milling with glass beads (0.25-0.40 mm), resulted in 99% disruption of the biomass cells; whereas for *Phaeodactylum tricornutum,* bead milling with glass beads (0.25-0.40 mm), gave 75% disruption. In a following experiment, in which Zr beads (0.3mm) were used, the maximum disruption efficiency we achieved was approximately 13% higher for *Phaeodactylum tricornutum*. In the present study, we also show that fat and soluble protein release, increased by bead milling the microalgae biomass; while by bead milling, we also reduced the aerobic bacteria content of the processed biomass.

Electropermeabilization treatment of the above microalgae biomasses with the use of Pulsed Electric Fields (PEF), as an alternative to conventional cell disruption methods was also tested. Electropermeabilization, the phenomenon in which, the cell membrane becomes permeable due to electricity, is affected by different PEF treatment parameters. In the present study, field strength of 1 and 3 kV/cm was applied, for extraction of bioactive compounds and measurement of total antioxidant capacity of *T. chuii* and *P. tricornutum* using different solvent (H_2O and DMSO) and extraction time (4h and 24h). PEF treatment, extraction solvent and time affected significantly the tested variables (analyzed levels of chlorophyll a and b, total carotenoids, total antioxidant capacity and phenolic compounds) for both microalgae; with the exceptions of phenolic compounds released from *P. tricornutum*, and chlorophyll b released from *T. chuii*. Extraction of phenolic compounds was affected significantly by solvent and the interaction of solvent and PEF treatment. Last, the extraction levels of chlorophyll b were significantly affected by PEF treatment, extraction solvent and the interaction of extraction time and solvent.

Keywords: *Tetraselmis chuii, Phaeodactylum tricornutum, cell wall disruption, bead milling, pulse electric fields (PEF), chlorophyll, carotenoids, soluble proteins, EPA, DHA, phenolic compounds, total antioxidant capacity.*

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List of abbreviations

AACC	American Association of Cereal Chemists
AU	Absorbance Unit
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
AOCS	American Oil Chemists' Society
ARA	Arachidonic Acid
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
CCD	Central Composite Design
DHA	Docosahexaenoic Acid
DM	Dry matter
DMRT	Duncan's Multiple Range Test
DW	Dry Weight
EAA	Essential Amino Acids
EC	European Commission
EPA	Eicosapentaenoic Acid
FAME	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed Conversion Rate
FID	Flame Ionization Detector
FM	Fish Meal

FO	Fish Oil
FR	Flow Rate
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
GLA	Gamma Linoleic Acid
GLC	Gas Liquid Chromatography
GLM	General Linear Model
GM	Genetically Modified
GMO	Genetically Modified Organisms
GRM	General Regression Model
i.e.	in example
LC	Long Chain
NAM	National Algaepilot Mongstad
NOFIMA	The Norwegian Institute of Food, Fisheries and Aquaculture Research
OECD	The Organisation for Economic Co-operation and Development
ORAC	Oxygen radical absorbance capacity
p.a.	Per annum
PEF	Pulse Electric Fields
PhD	Doctor of Philosophy
ppm	parts per million
ppt	parts per thousands
PUFA	Polyunsaturated fatty acid
SDS	Sodium dodecyl sulfate

SEM	Scanning Electron Microscope
SEM	Standard Error of Means
SM	Stress Model
TAC	Total Antioxidant Capacity
TE	Trolox Equivalent
TEAC	Trolox Equivalent Antioxidant Capacity
TPC	Total Phenolic Compounds
TS	Tip Speed
v/v	volume per volume
WR	Working Reagent

1.0 Introduction

1.1 The modern aquaculture

Aquaculture is one of the fastest growing food sectors and will continue being that despite its average annual growth rate slowing down from 5.4% p.a. in the previous decade to 3.0% p.a. in the period 2016-2025 (OECD, 2016). The term aquaculture encompasses the farming of all aquatic organisms, i.e., fish, mollusks, crustaceans, and macro/micro algae. Farming implies some form of intervention in the growing process, such as regular stocking, formulation of feeds, controlled breeding, etc., in order to enhance production. Aquaculture fish and crustaceans are reared in high-density systems and cannot forage freely on natural food. Thus, they must be provided a diet which supplies all the necessary ingredients (protein, carbohydrates, fats, vitamins, minerals and pigments) for optimal growth and health. Fishmeal and fish oil, mostly coming from wild caught pelagic fish, are key ingredients used in feed production for aquaculture, providing the organisms with the essential amino and fatty acids, which they cannot synthesize themselves; creating though dependence on wild fish stocks (Ytrestøyl *et al.* 2015; Aas *et al.*, 2019). Modern feed technology combines fish meal and oil with other ingredients, like soya protein, wheat gluten, krill hydrolysates, rapeseed oil, pigments (carotenoids synthetic and natural), vitamins and minerals, and produces diets specialized for each species (Martin *et al.* 2017).

1.2 The need for new sustainable feed ingredients

In finfish aquaculture, even though there have been advances in feed technology, and plants have replaced some of the proteins and lipids needed, there is still a significant dependency on forage fish, with more than 1 kg wild fish equivalents needed to produce 1 kg of carnivorous farmed fish, making their aquaculture unsustainable (Shepherd & Jackson 2013; Oliva-Teles *et al.* 2015). Small pelagic species are caught and subjected to several processes to produce fish oil and fish meal, which are mainly used as ingredients in feeds for aquaculture systems (Kuah *et al.,* 2015). The fast-growing sector of aquaculture, demands more and more raw materials, increasing the fishing pressure on wild stocks which supply fish meal and fish oil, threatening their sustainability (FAO 2016). Fish oil production worldwide, is more than one million tons annually and only the salmon feed industry utilizes about 50 % of this (Naylor *et al.,* 2009; Shepherd & Jackson 2013). FAO implies that our dependence on fishmeal for aquaculture is undermining both marine biodiversity and human food security (FAO, 2018). Undoubtably, establishment of sustainable alternative feed ingredients to fish meal and fish oil is vital. The need for alternative sources of nutrients, especially containing essential long chain polyunsaturated ω-3 fatty acids (i.e. EPA and DHA), has led us to exploiting other alternatives like zooplankton, mesopelagic fish, by-catch/by-products and microalgae, insects, as well as genetically modified (GM) plants (Sissener *et al.,* 2011; Oliva-Teles *et al.,* 2015; Henry *et al.,* 2015; Napier *et al.,* 2015). Zooplankton such as krill and calanoid copepods, are a good oil source, but the technological challenges, the harvesting costs, and the danger that lies on fishing down the marine food web, makes zooplankton a controversial alternative (Tocher 2015). Future use of

mesopelagic fish, like lanternfish and myctophids, as fish meal and fish oil source, even though available in potentially large quantities (Irigoien *et al.,* 2014), will not bring a change to the fish to fish principle governing fin fish aquaculture. GM plants are produced widely and are approved for use in fish feeds both in Norway and the European Union (Regulation (EC) 1829/2003). However, GMO is a controversial matter for society, and consumers, especially in Europe, are skeptical to GM ingredients (Wesseler, & Kalaitzandonakes 2019). Thus, because Norwegian fish farmers do not wish any doubts on their product, they are not using feeds which contain GM ingredients (Sørensen *et al.,* 2011). Microalgae are a promising, even currently less exploited due to high production costs, and potentially sustainable source of nutrients (Madeira *et al.* 2017). Apart from being a good ω-3 fatty acid source (Kumar *et al.* 2019), microalgae are rich in high quality proteins and high value compounds, such as vitamins, pigments, phenolics, and other bioactive substances (Madeira *et al.,* 2017; Shah *et al.,* 2018; Yarnold *et al.,* 2019).

1.3 Microalgae

Microalgae are unicellular photosynthetic microorganisms, living both in marine and freshwater environments. They form the basis of the ecosystem's trophic pyramid, as they are primary producers of essential nutrients and contain important amounts of nutrients, such as proteins, long chain ω-3 polyunsaturated fatty acids, vitamins, carbohydrates, antioxidants, pigments, and minerals. Traditionally, live microalgae have been used as nourishment for larval and juvenile stages in aquaculture, but when it comes to adults, microalgae are less exploited assumedly due to high production costs (Molina

Grima *et al.,* 2003). Some of the most frequently used microalgae in aquaculture are species belonging to the genera *Chlorella, Tetraselmis, Isochrysis, Arthrospira, Phaeodactylum, Haematococcus, Nannochloropsis,* and *Schizochytrium* (Kaparapu 2018; Garrido-Cardenas *et al.,* 2018). Commercial, large-scale and lower cost production of biomass is necessary should microalgae become a realistic alternative to fish meal and fish oil in aquaculture feeds. Introducing microalgae biomass in aquafeeds will render aquaculture more sustainable reducing the ecological impact of the sector (Muller-Feuga, 2000; Shah *et al.,* 2017).

1.4 Microalgae nutritional value

1.4.1 Fats and fatty acids

In general, oil content in microalgae can surpass 60% by weight of dry biomass, while levels of 20– 50% are the most common (Guschina and Harwood 2013). Microalgal lipids contain polyunsaturated fatty acids such as DHA (*Schizochytrium sp.*), and EPA (*Nannochloropsis sp., Phaeodactylum sp., Isochrysis sp.*). Polyunsaturated fatty acids (PUFAs), like gamma-linolenic acid (GLA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) arachidonic acid (ARA) are generally considered essential fatty acids, having a multifunctional role in an organism. Apart from their nutritional value, they play key roles in several physiological functions including maintenance of cardiovascular, immunity and anti-inflammatory responses, and neurological health (Brodtkorb et al., 1997; Calder et al., 2004;2006).

In the aquaculture industry, long chain ω -3 polyunsaturated fatty acids (ω-3 LC-PUFA) are currently obtained from fish oil and are considered a limiting factor as they are absent in vegetable oils such as palm, soybean, and rapeseed/canola oils also used in aquafeed (Shah et al., 2017). Microalgae are considered among the most prominent future sustainable sources of ω-3 LC-PUFA-rich oils (Taelman *et al.,* 2013). Generally, fish oil replacement by microalgae and other microalgae like unicellular organisms (e.g. *Thraustochytrids*) has been an object of research with positive results both for the growth of the organisms and the quality of the produced product. A typical example is the study of Kousoulaki et al., (2016) in which spray dried *Schizochytrium sp.* biomass was included up to 5% in extruded feeds for salmon, successfully replacing fish oil as source of ω-3 LC-PUFA without compromising fish growth rate and FCR, dietary protein and energy digestibility and filet flesh quality. In the same study, they concluded that dietary *Schizochytrium sp*. improved the retention efficiency of EPA, DHA and monounsaturated fatty acids of salmon fillet.

1.4.2 Proteins and amino acids

The nutritional value of protein is determined by the content and availability of its constituent amino acids. The amino acid composition of microalgae is quite similar to chicken egg protein, considered of high nutritional value for humans, although the latter is richer in methionine and lower in arginine (Teshima *et al.,* 1986). Some amino acids are unavailable for animal digestion and absorption if sections of the molecule are bound to other molecules (e.g. the free amino group of lysine can sometimes be bound to carbohydrate, particularly, during processing of harvested algae (like drying)). The

essential amino acid (EAA) profiles of *T. chuii* and *P. tricornutum* are similar (Kokkali *et al.*,2018a). The comparison of the above microalgae with high-quality protein rich plant and marine raw materials, used in fish feed, showed some noteworthy differences. For instance, *T. chuii* and *P. tricornutum* contain higher % of nearly all EAA in their protein compared to wheat gluten, but only higher levels of methionine compared to soy protein concentrate, whereas fish meal had higher relative to protein levels of histidine, arginine, methionine, leucine and lysine and lower levels of threonine, valine, isoleucine and phenylalanine (Kokkali *et al.*,2018a).

1.4.3 Micronutrients and bioactive compounds in microalgae

1.4.3.a Vitamins

Algae are a significant source of nearly all the vitamins. However, few studies have been conducted on marine micro-algae and all of them are more than two decades' old (Kanazawa 1969, Aaronson *et al.*, 1971, Brown *et al.*, 1999). The major vitamins identified in microalgae are thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxine (vitamin B6), cyanocobalamin (B12), biotin (vitamin H), ascorbic acid (vitamin C), nicotinic acid (vitamin B3), pantothenic acid (vitamin B5), choline (vitamin B4), inositol (vitamin B8), tocopherol (vitamin E) and ß-carotene (provitamin A), vitamin K and vitamin D. Microalgae vitamin profile depends on microalgae species, genotype, growth phase and the nutritional status of the algae (cultivation method, starvation, limitations) (Brown *et al.*,. 1999). Noteworthy, heat unstable vitamins, like thiamine, riboflavin, ascorbic acid, nicotinic acid, could be considerably affected by techniques which demand temperature increase (i.e. drying).

Some vitamins found in algae biomass can also derive from bacteria grown together with the algae. A clear example is the presence of vitamin B12 in *Chlorophyceae* and *Rhodophyceae*; as it was accepted that these algae classes were not able to synthesize vitamin B12 (Becker 2004).

1.4.3.b Pigments

Animals lack the ability to synthesize pigments endogenously and thus obtain these compounds *via* their diet. The major pigments of most algae are the green chlorophylls and the yellow, orange and red carotenoids, which amount up to 0.5-5% of the dry weight of the cell (Parsons *et al.*,1961; Ben-Amotz *et al.*,1985). Blue-green algae, red algae and the cryptophytes also contain the red, protein- bound and water-soluble phycoerythrin and/or the blue phycocyanin. Chlorophylls and carotenoids follow the extracted lipid fraction of the processed biomass. Carotenoids are made up of a number of isoprene units, functioning both as photoprotectants and light-harvesting pigments in photosynthesis (Cohen 1986). Each algal species may contain between 5 and 10 different carotenoids, and more than 60 different carotenoids are known from algae (Cohen 1986). ß-carotene, is a common constituent of the carotenoid fraction of microalgae, found in highest concentration in the green algae.

Pigments play a key role in aquaculture, as they are added in the fish feeds to enhance skin and fillet coloration of some farmed species (Amaya & Nickell, 2015). Atlantic salmon, rainbow trout, red and gilthead sea bream, red tilapia, Pacific white shrimp are some species in which there is an extensive use of pigments, such as carotenoids, in their feeds in order to obtain a vivid skin and in some cases, i.e., Atlantic

salmon, filet coloration (Wade *et al.*, 2017; Betsy & Kumar, 2018; Pérez -Legaspi *et al.*, 2019). *Nannochloropsis sp*. is a well-known source of different valuable pigments, such as chlorophyll a, zeaxanthin, canthaxanthin, and astaxanthin. *Phaeodactylum sp*. is rich in fucoxanthin, carotene, chlorophyll a, and chlorophyll c, while *Tetraselmis sp*. contains chlorophyll a and b, β-carotene, zeaxanthin, and violaxanthin. *Chlorella vulgaris* contains fucoxanthin, zeaxanthin, and lutein (Shah *et al.*, 2017), and *Haematococcus pluvialis* contains β-carotene, astaxanthin, canthaxanthin and lutein (Choubert and Heinrich, 1993). Among all, *Haematococcus pluvialis* can play a leading role in replacement of synthetic pigments in fish feeds, as it produces large amounts of astaxanthin, the key pigment giving the pink color of salmon and trout fillets (Shah *et al,* 2016). In addition to salmon feeds, *Haematococcus pluvialis* has been tested successfully also in other species, such as shrimps. Namely, Pacific white shrimp fed a diet where 12.5% of dietary fish meal was replaced by dry microalgae meal (by product from dried *Haematococcus pluvialis* biomass), had improved pigmentation compared to the control without and there was no other detected negative effect on the animal's overall performance (Ju, Deng and Dominy, 2012).

1.4.3.c Bioactive compounds

Bioactive compounds are physiologically active substances with essential attributes for an organism. Apart from macronutrients which may have bioactive action (i.e. PUFAs and amino acids), microalgae also contain bioactive compounds such as β-glucans, β-carotenes, polyphenols, sterols, flavonoids, phycobiliprotein, nucleotides and water-soluble peptides. These bioactive compounds can be either obtained from the

microalgae biomass or found in the culture medium, after extracellular release into it (Bhagavathy *et al.,* 2011).

Studies have shown that there are several microalgae containing bioactive substances with medicinal properties (de Morais *et al.*,2015). The antioxidant and anti-inflammatory effect of *T. chuii* methanolic extracts, which have potent nitric oxide inhibitors, was indicated by a study of Banskota *et al.*, (2013). Experimental studies on Chlorella demonstrated antitumor, anticoagulant, antibacterial, and antioxidant effect; while antioxidants such as lutein, α- carotene, β-carotene, ascorbic acid, and α-tocopherol, which act against free radicals, were also identified (Cha *et al.*,2010; Li *et al.*, 2010). The xanthophyll fucoxanthin, which is a carotenoid found in numerous microalgae classes, has shown a great antioxidant activity, anti-cancer, anti-diabetic and anti-photoaging properties (Peng *et al.*,2011). *P. tricornutum* is considered as one of the primary commercial sources of another carotenoid pigment, the xanthophyll fucoxanthin, as it can produce more than 1.5% of fucoxanthin in dry weight (Yi *et al.*, 2015). Koo et al. (2019), after experimenting the anti-obesity effects of a powder produced as a commercial functional food by *P. tricornutum* microalgae in mice, concluded that the *Phaeodactylum* extract, which contains fucoxanthin, exerts anti-obesity effects by promoting lipolysis and inhibiting lipogenesis.

Apart from their potential effect on human health, bioactive compounds from microalgae could enhance the well-being of fish by improving gut health and thus nutrient assimilation and immune competence and thus resistance to pathogens and disease (Shah *et al.*, 2017). Prominent gut immune modulating were observed in Atlantic salmon fed increasing levels of *Schizochytrium sp.* in the diet (Kousoulaki *et al.*, 2015).

In this study, salmon fed *Schizochytrium sp.* enriched diets exhibited strong innate immune responses, such as increased number of Goblet cells, without signs of intestinal irritation that in another case could also justify increased mucus production.

1.5 Downstream processing of microalgae

Biorefinery encompasses all the techniques used to process the microalgae, and retrieve the high value nutrients from them, for nutritional, pharmaceutical and chemical applications. Cell wall disruption, pre-treatment of the biomass with extraction enhancing techniques, effective drying, biomass stabilization, are some of the most common techniques applied on microalgae biomass for downstream processing (Gilbert-López *et al.,* 2015; Sahoo *et al.,* 2017; Khanra *et al.,* 2018).

Monogastric animals cannot digest microalgae due to their hard-cellulosic cell walls; specifically, studies have shown that carnivorous fish, like Atlantic salmon (*Salmo salar)* (Sørensen *et al.*,2016), and hens (Lemahieu *et al.*,2016) utilize more efficiently nutrients from cell wall disrupted microalgae. Thus, cell wall disruption is indispensable for the efficient release of nutrients from some microalgae biomasses (Phong *et al*., 2018). Microalgae cell wall disruption can be achieved with various methods such as bead milling, high pressure homogenization, microwaves, freezing, pulse electric field (PEF) (Günerken *et al.,* 2015; Lee *et al.,* 2017); but the effectiveness of each method varies, and the potential of industrial implementation is a key for the selection of the most appropriate, cost efficient method.

1.5.1 Cell wall disruption

There are several methods used in order to achieve microalgae cell wall disruption, including mechanical and non-mechanical ones (Table 1). The mechanical methods are divided in three categories:

1) those applying solid shear, as for instance bead milling and high-speed homogenisation,

2) those applying liquid shear, as for instance high-pressure homogenisation and ultrasonication,

3) and other, such as microwaves and pulsed electric field (PEF).

The non-mechanical methods used for microalgae or bacterial cell wall disruption can be chemical or enzymatic.

Table 1: Unicellular organisms cell wall disruption method. Table adapted from Günerken *et al.*, 2015.

Cell wall disruption methods					
Non mechanical					
Chemical			**Enzymatic**		
Mechanical					
Solid Shear		Liquid Shear		Other	
HSH High speed homogenization	**Bead milling**	**HPH** High pressure homogenization	**Ultrasound**	**Microwave**	**PEF** Pulse electric fields

In a recently published study on cell wall disruption of *Chlorella vulgaris* (Postma *et al., 2*016), the authors observed that induction of cell permeabilization by application of pulse electric field (PEF) allowed greater release of small soluble components, whereas carbohydrates were also released more efficiently with simultaneous application

of heat and PEF. Nevertheless, other nutrients such as proteins were still highly retained, but could be effectively released by bead milling, either alone or following PEF.

The use of enzymes, such as cellulose, lysozyme and snailase can result in effective cell wall disruption too (Fu *et al.*,2010). However, this process implicates additional costs on enzymes, and heating to 37-55 ^{o}C during reaction and even higher temperature for enzyme inactivation, which may cause degradation of the final product (i.e. protein denaturation).

A more recent study by Safi et al. (2017) on cell wall disruption of *Nannochloropsis gaditana* shows that high-pressure homogenization and bead milling are the most efficient methods compared to enzymes and PEF for efficient protein release from microalgae. In a comparative study by Zheng et al. (2011) the most efficient cell wall disruption method for *Chlorella vulgaris* was manual grinding in a ceramic mortar under liquid nitrogen. However, this method is not practical or easily up scalable. Unlike in Postma et al. (2016) and Safi et al. (2017), the Zheng et al (2011) study showed low efficiency of nutrient release by bead milling, and the reason may lay in the fact that in the latter study were applied different processing parameter settings. Bead milling has many operating parameters such as bead type and diameter, bead density in the milling chamber, agitator speed and chamber flow rate (Montalescot *et al.,* 2015; Garcia *et al.*,2019); which affect differently the disruption efficiency.

Table 2 lists some microalgae cell wall disruption by bead milling studies, and the different parameters that were used. The variety of parameters and the different results obtained, makes clear the need for optimization of the cell wall disruption processing for each different method applied.

1.5.2 Drying

Part of the microalgae biorefinery processes is drying of the microalgae biomass slurry (15-30% dry matter), in order to simplify logistics and use, and extend the shelf life of the product. Some of the most common drying methods include sun drying, freeze drying, spray drying. The cost of drying process increases significantly the overall processing cost and requires significant energy. Even though sun drying is admitted as a cheap method, drawbacks such as long time and uneven drying, and risk of material loss (Prakash *et al*.,1997) make it less attractive. On the other hand, spray drying is supposed the most efficient drying method, especially for products targeting human consumption (Chen *et al.,* 2011). Nevertheless, studies have shown that significant deterioration of some algal pigments (Desmorieux and Decaen 2005) and lipids (Villagracia *et al.*,2016) can be caused during spray drying, most likely due to the high temperatures involved in this process. Freeze-drying, or lyophilization, is a widely used method for drying microalgae in research level; but may be too costly for some larger-scale applications.

Table 2: Literature review of bead milling studies with different bead milling parameters.

Species	Dry matter (g/l)	Flow rate (kg/h)	Bead type	Bead size (mm)	Bead filling (%)	Agitator speed (m/s)	Champer volume (L)	Disruption (%)	Response parameter	Reference
									Bacteria reduction (%)	
Chlorella sp.	112	10.2	glass	0.15-0.25	82	10	1.4	55	67.6	Doucha & Lívanský (2008)
Chlorella sp.	112	15	glass	0.15-0.26	82	14	1.4	70	73.1	" "
Chlorella sp.	107	20	glass	0.2-0.3	82	14	1.4	92.5	78.7	" "
Chlorella sp.	107	3	glass	0.42-0.58	82	10	1.4	99	85.7	" "
Chlorella sp.	107	5	glass	0.42-0.58	82	10	1.4	96.5	83.8	" "
Chlorella sp.	107	8	glass	0.42-0.58	82	10	1.4	96.5	79.8	" "
Chlorella sp.	107	3	glass	0.42-0.58	82	14	1.4	99.9	90.2	" "
Chlorella sp.	107	5	glass	0.42-0.58	82	14	1.4	99	87.9	" "
Chlorella sp.	107	15	glass	0.42-0.58	82	14	1.4	97	84.2	" "
Chlorella sp.	107	40	glass	0.42-0.58	82	14	1.4	90	76.2	" "
Chlorella sp.	112	10.2	glass	0.42-0.58	82	10	1.4	80	75.3	" "
Chlorella sp.	112	10.2	glass	0.42-0.58	82	14	1.4	92	78	" "
Chlorella sp.	102.5	3	ZrO_2	0.3	85	14	1.4	92.3	84.3	" "
Chlorella sp.	102.5	10	ZrO_2	0.3	85	14	1.4	83.2	80.9	" "
Chlorella sp.	102.5	15	ZrO_2	0.3	85	14	1.4	77.9	76.7	" "
Chlorella sp.	69.4	5	glass	0.3-0.4	85	-	0.6	95	98.6	" "
Chlorella sp.	69.4	10	glass	0.3-0.4	85	-	0.6	93	96.9	" "
Chlorella sp.	69.4	10	glass	0.3-0.4	85	-	0.6	98-99	99.5	" "
Chlorella sp.	69.4	10	glass	0.3-0.4	85	-	0.6	98-99	99.2	" "
Chlorella sp.	69.4	4.7	glass	0.5-0.7	85	-	0.6	96	99	" "
Chlorella sp.	69.4	12	glass	0.5-0.7	85	-	0.6	92	98.7	" "
Chlorella sp.	69.4	18	glass	0.5-0.7	85	-	0.6	70	95.3	" "
Chlorella sp.	69.4	5	glass	1.0-1.2	85	-	0.6	93-94	96.6	" "
Chlorella sp.	69.4	9	glass	1.0-1.2	85	-	0.6	91-92	92.6	" "
Chlorella sp.	141.7	5	glass	0.5-0.7	85	-	0.6	70	89	" "
Chlorella sp.	141.7	10	glass	0.5-0.8	85	-	0.6	64	69.5	" "
Chlorella sp.	141.7	10	glass	0.5-0.9	85	-	0.6	71.5	91	" "
Chlorella sp.	141.7	10	glass	0.5-1	85	-	0.6	83	-	" "
Chlorella sp.	-	10	glass	0.3-0.4	85		0.6	98-99	-	Günerken *et al.* (2015)
Tetraselmis sp.	-	1.5*	ceramic	0.3-0.4-0.6	65		0.3	**	Protein release	Günerken *et al.* (2015)
Chlorella sp.			glass	0.4-0.6			240		Total lipid release	Zheng et al. (2011)
N. gaditana	100		ZrO_2	0.5	65	8			Protein release	Safi *et al.* (2017)

* L/min ** 21% of protein transferred to algae extract

1.5.3 Goal of the current study

In the current study we worked towards optimizing cell wall disruption of *Tetraselmis chuii* and *Phaeodactylum tricornutum*, for efficient release of macronutrients and bioactive compounds. Bead milling, as well as Pulsed Electric Fields, were the two disintegration methods applied. Cell cytometry was the main method used for the determination the disruption efficiency of the used processing methods. Various other analyses were also performed either as additional means for the evaluation of disruption efficiency, for determination of the nutrient profile and the bioactive compound content of the aforementioned microalgae; as well as for learning and standardizing protocols and methods in the host institutes' laboratories. Retention of the processed biomass, and preservation of the quality of macronutrients and bioactive compounds, by drying of the product was also performed. The expected results of this study were the definition of optimal processing parameters of different microalgae species biomass for the release of essential and valuable nutrients, such as lipids and proteins, in order to become available for the digestive system of *Atlantic salmon*, as well as for the production of algae-based bread and beer products.

2.0 Materials and Methods

2.1 Microalgae samples' origin, and morphology

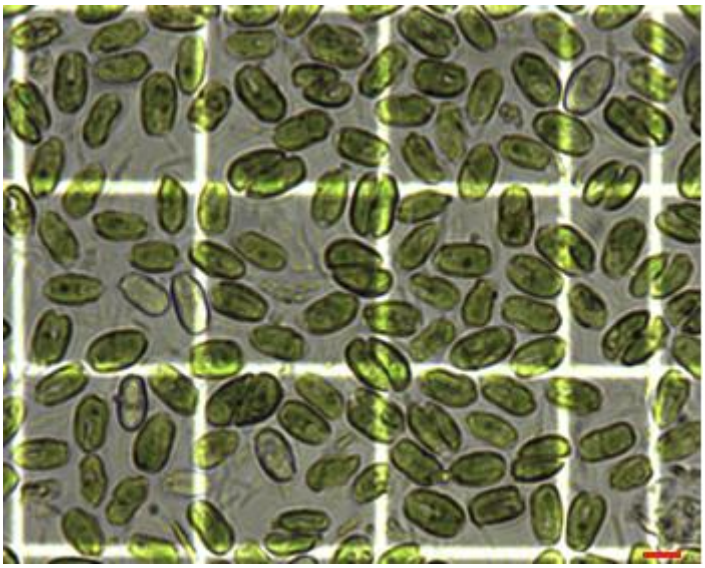
Tetraselmis chuii (Figure 1a) and *Phaeodactylum tricornutum* (Figure 1c) were produced in four 800L GemTube (LGEM, The Netherlands) photobioreactors at the National Algaepilot plant in Mongstad (NAM) north of Bergen, Norway. The photobioreactors were located in a greenhouse exposed to natural light and additionally equipped with artificial illumination (EAX 170W LED lights, Evolys AS, Norway) with an average incident artificial light of 200 µmol m^{-2} s^{-1}. The *Phaeodactylum tricornutum* (*P. tricornutum*) biomass used in the studies of this thesis was produced in May and June 2017, whereas *Tetraselmis chuii* (*T. chuii*) biomass was produced in July – October 2017. The reactors were operated at pH 7.8 by on-demand CO_2 addition, and culture temperatures were maintained between 15 and 35°C by heating the greenhouse, or spraying the reactors with water, to cool down. The reactors were operated in dual mode, as such mixing was provided by both liquid pump and air pump, resulting in a liquid velocity of approximately 0.3 m s^{-1}. The microalgae were cultivated in modified WUR medium, which was based on natural seawater (Fensfjorden, Mongstad, salinity of 31 ppt), enriched with a nutrient stock solution (Table 3). Seawater was chemically sterilised (sodium hypochlorite), active chlorite was deactivated by filtration through active carbon, followed by filtration (1 µm).

Table 3: Medium mineral concentration.

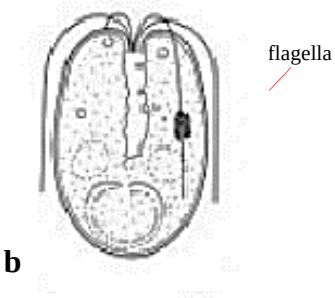
Mineral final consentration (in mM)					
$NaNO_3$	25	NA_2EDTA	0.56	$MnCl_2.H_2O$	0.01
KH_2PO_4	1.7	Fe_2SO_4	0.11	$ZnSO_4 \cdot H_2O$	$2.3 \cdot 10^{-3}$
$Co(NO_3)_2 \cdot 6H_2O$	$0.24 \cdot 10^{-3}$	$CuSO_4 \cdot 5H_2O$	$0.1 \cdot 10^{-3}$	$Na_2MoO_4 \cdot 2H_2O$	$11 \cdot 10^{-3}$

The microalgae biomass was produced in a fed-batch process: the reactors were harvested once per week (between 50-90% of the culture volume), after which seawater and nutrients were added to compensate for the volume taken. After harvesting, the biomass was dewatered using a spiral plate centrifuge (Evodos 25, Evodos b.v., The Netherlands), resulting in paste of approx. 22% dry weight in case of *P. tricornutum* and approx. 35% dry weight in case of *T. chuii*. The paste was vacuum packed and directly frozen at -20°C before sending to Nofima in Bergen, Norway and stored at -20ºC until further use.

T. chuii is a marine unicellular microalgae 12-14µm in length, 9-10µm in width and belongs to the family *Chlamydomonadaceae*. Characteristic of the species is the ovoid shape of the cell and the four flagella which emerge from a depression near the apex (Figure 1b). *P. tricornutum* is a marine diatom 5-27µm in length, 3-4µm in width and belongs to the family *Phaeodactylaceae*. *P. tricornutum* can be found in different morphotypes (Figure 1d) (i.e. fusiform, triradiate, and oval). Triradiate morphotype is characteristic of the *Atlantic* strain. All *P. tricornutum* morphotypes were observed in our samples under the microscope.



a



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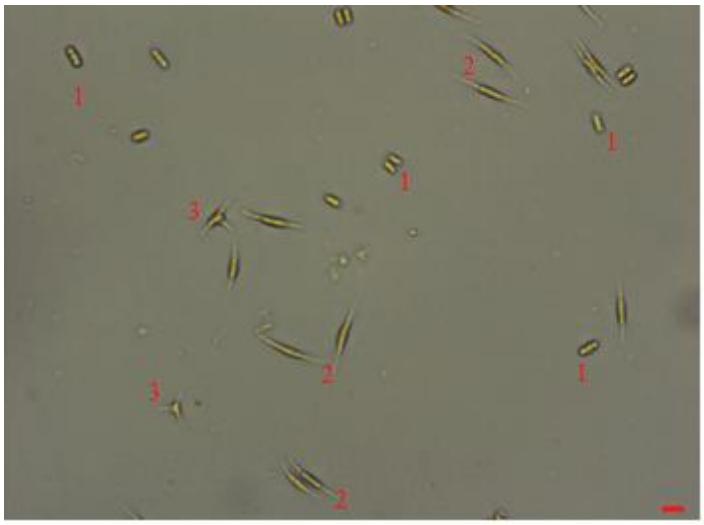

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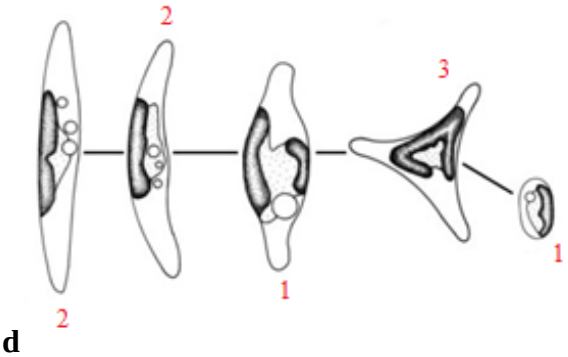

d

Figure 1: Microalgae morphology and characteristics.
(a*) T. chuii cells observed on an optical microscope (Photo: Kokkali)* Scale bar 10µm *(b) T. chuii cell morphology. Illustration from Andersen (2013). (c) P. tricornutum cells observed on an optical microscope (Photo: Kokkali).* Scale bar 10µm, *(1: oval, 2: fusiform, 3: triradiate) (d) P. tricornutum morphotypes (1: oval, 2: fusiform, 3: triradiate). Illustration from Andersen (2013).*

2.2 Cell wall disruption of microalgae biomasses by bead milling

2.2.1 Microalgae pre-processing for bead milling

For each processing trial we produced biomasses of five different dry matter (DM) concentrations by dilution of the thawed concentrated biomass paste batches with the necessary amounts of tap water (Table 4**Error! Reference source not found.**). DM w as estimated before and after dilution of the algae paste batches by a HG53 Mettler Toledo, Moisture Analyzer, in order to calculate the necessary amount of water to be added and to verify that the desired DM content was reached.

Table 4: Different Dry matters for each experimental trial.

Trial	Biomass	Final dry matter (%)				
1: Tetraglass	*Tetraselmis chuii*	10	15	17.5	20	22.5
2: Phaeoglass	*Phaeodactylum tricornytum*	13.65	15.77	18.89	22	24.12
3: PhaeoZr	*Phaeodactylum tricornytum*	11.78	14	17.25	20.5	22.72

2.2.2 Bead milling

A Dyno-Mill Multi Lab (WAB, Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) (Figure 2), was used for the microalgae cell wall disruption, which consisted of a horizontal milling chamber filled with beads, a central shaft with five agitator discs, and a pump which is victualing the chamber. The beads are accelerated in the bead mill chamber in a radial direction across the shaft and the aggregator speed was set manually on different tip speed (TS) between 6.5 and 12.1 m/s in each trial set up (Table 5).

Figure 2: Dyno-Mill Multi Lab (WAB). *(Photo: Kokkali)*
a)The agitator bead mill with with 0.6 l grinding container and digital display. b) The agitator discs mounted on the agitator shaft c)1: unprocessed biomass mixing, 2: pump which "feeds" the mill, 3: product inlet, 4: 0.6 l grinding container (chamber), 5: product outlet, 6: disrupted biomass collected on cooled.

Table 5: Different Tip speeds used in the different experimental trial.

Trial	Biomass	Tip Speed (m/sec)				
1: Tetraglass	*Tetraselmis chuii*	8	9	10	11	12
2: Phaeoglass	*Phaeodactylum tricornytum*	7.98	8.8	10	11.2	12.02
3: PhaeoZr	*Phaeodactylum tricornytum*	6.6	7.2	8.1	9	9.6

The biomass feeding pump was set on three different algae biomass flow rates (FRs) (1, 2 and 3), however, possibly due to the difference in viscosity and density of the

different biomasses, the achieved FR varied more (Figure 3a, Figure 3b) . The actual FR were calculated by measurement of produced biomass on a specific time frame; and only the actual FR values were used in the statistical analyses of the data (Table 6).

For our experiments two different bead types were used; glass and zirconium (Zr). After bead milling of *T. chuii* biomass with glass beads, we achieved the maximum desired disruption; thus, for biomass thrift bead milling with Zr beads was not tested. For *P. tricornutum,* both bead types were used. Beads size was kept constant at 0.25-0.4 mm diameter for glass beads, and 0.3mm diameter for Zr beads. The chamber volume used was 0.6 l, and the beads were added at 80% chamber filling rate.

The bead mill was operated continuously during sampling of the differently processed batches of microalgae biomass. Based on chamber volume and filling, and the flow rate that was used, we calculated the collection and unload time between different experimental set ups. For instance, after collecting samples processed at a specific set of conditions, e.g. tip speed of 10 m/sec, and a flow rate of 100 ml/min, we then changed the processing parameters to the next set point, e.g. increasing the tip speed to 12 m/sec, we did not empty the chamber, but we waited for 3 minutes before we started collecting the new sample (using a 0.6 l chamber volume, at 80% chamber bead filling rate, the standing biomass in the chamber was measured to be ~120ml, and at chamber filling rate 100 ml/min; we estimated that all biomass processed at the previous set of conditions was removed after 2 min and 12 sec, which was the time we waited before collecting the new sample). During processing, the biomass samples were collected and either kept frozen at -20^{o}C for further analyses or were dried either by freeze or spray drying and stored at 4^{o}C. Freeze and spray drying procedures were executed by Nofima's personnel.

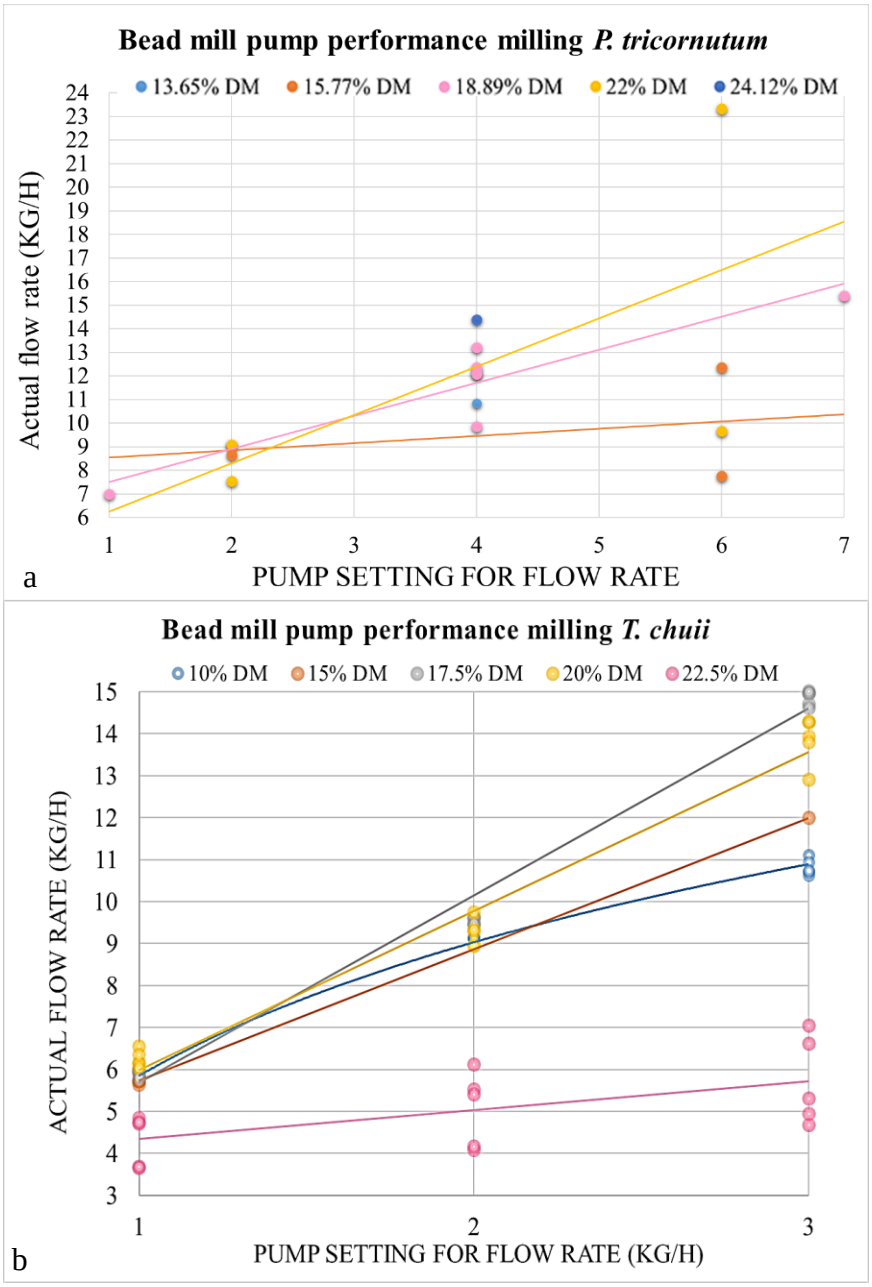

Figure 3: Pump setting and actual/measured flow rate of microalgae biomass during continuous bead milling of *Phaeodactylum tricornutum* (a) and *Tetraselmis chuii* (b).

Table 6 : Actual achieved Flow rates for each experimental trial.

Trial	Biomass	Flow rate (kg/h)										
1: Tetraglass	*Tetraselmis chuii*	5.8	6.0	6.5	8.9	9.3	9.5	9.7	12.9	13.9	14.3	14.6	15.0
2: Phaeoglass	*Phaeodactylum tricornytum*	7.0	7.5	7.8	8.7	9.0	9.9	10.8	12.1	12.4	13.2	14.4	15.4
3: PhaeoZr	*Phaeodactylum tricornytum*	5.7	8.8	9.0	9.2	13.1	13.4	13.5	13.7	14.7	22.1	22.5	22.8

2.2.3 Cytometry for the evaluation of cell wall disruption efficiency

To evaluate the cell wall disruption by bead milling freshly thawed samples were microscopically observed and cells of non-disrupted and disrupted samples of the same concentration were counted in known areas and compared. The samples were diluted with distilled water as shown on Table 7 and were vortexed for 1 min to obtain homogenous solutions. 20μl of the diluted samples were transferred, with the use of a positive displacement pipette (Pos-D™ MR-100, Mettler Toledo), in a Neubauer counting chamber and observed in a Nikon eclipse Ci optical microscope.

Table 7: Microalgae dilution, for microscopical observation.

	Microalgae sample (μl)	H_2O (μl)
Tetraselmis chuii	20	400
Phaeodactylum tricornytum	20	1780

In the Neubauer counting chamber the number of cells in the liquid sample, was counted within an approximate 22700 μm^2 area, 3x3 squares (Figure 4 and 5), and NIS Elements BR 4.40.00 software was used for analysis and photodocumentation of the samples . The reference points for the calculation of the disruption degree in the processed samples were non-disrupted biomass samples of the same Dry matter, and the %Disruption efficiency was calculated as in equation 1. For instance, Figure 4, illustrates in white, the Neubauers' guiding grid, in red, the marked counting area (3x3 squares), and inside this area, cross-marked with red color the intact cells; in the yellow box the area of the red square, and the number of cross-marked cells are shown. In Figure 4a (non-processed sample) we see 211 intact cells, in Figure 4b (bead milled sample) 93 intact cells; using equation 1, we calculate that the Disruption degree in this sample is 40.27%.

Eq.1 **%Disruption =**

$$= \frac{[(Average\ cell\ number\ in\ non\ disrupted\ sample) - (Average\ cell\ number\ \ in\ disrupted\ sample)] \times 100}{number\ of\ cells\ \ in\ non\ disrupted\ sample}$$

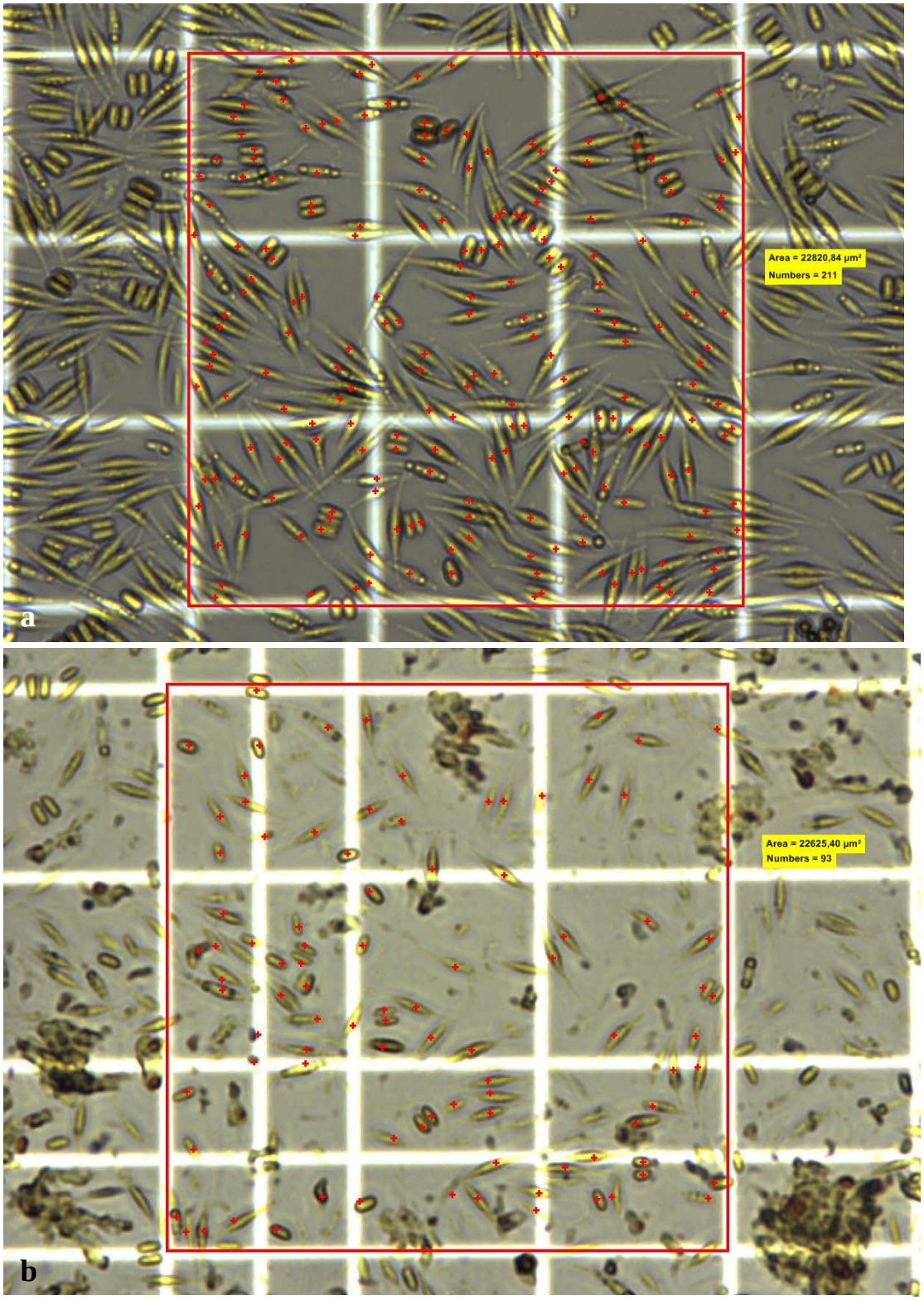

Figure 4: Neubauer squares as shown in the microscope, while counting.
P. tricornutum (initial DM 22%) non-disrupted (a) and disrupted (b) cells.

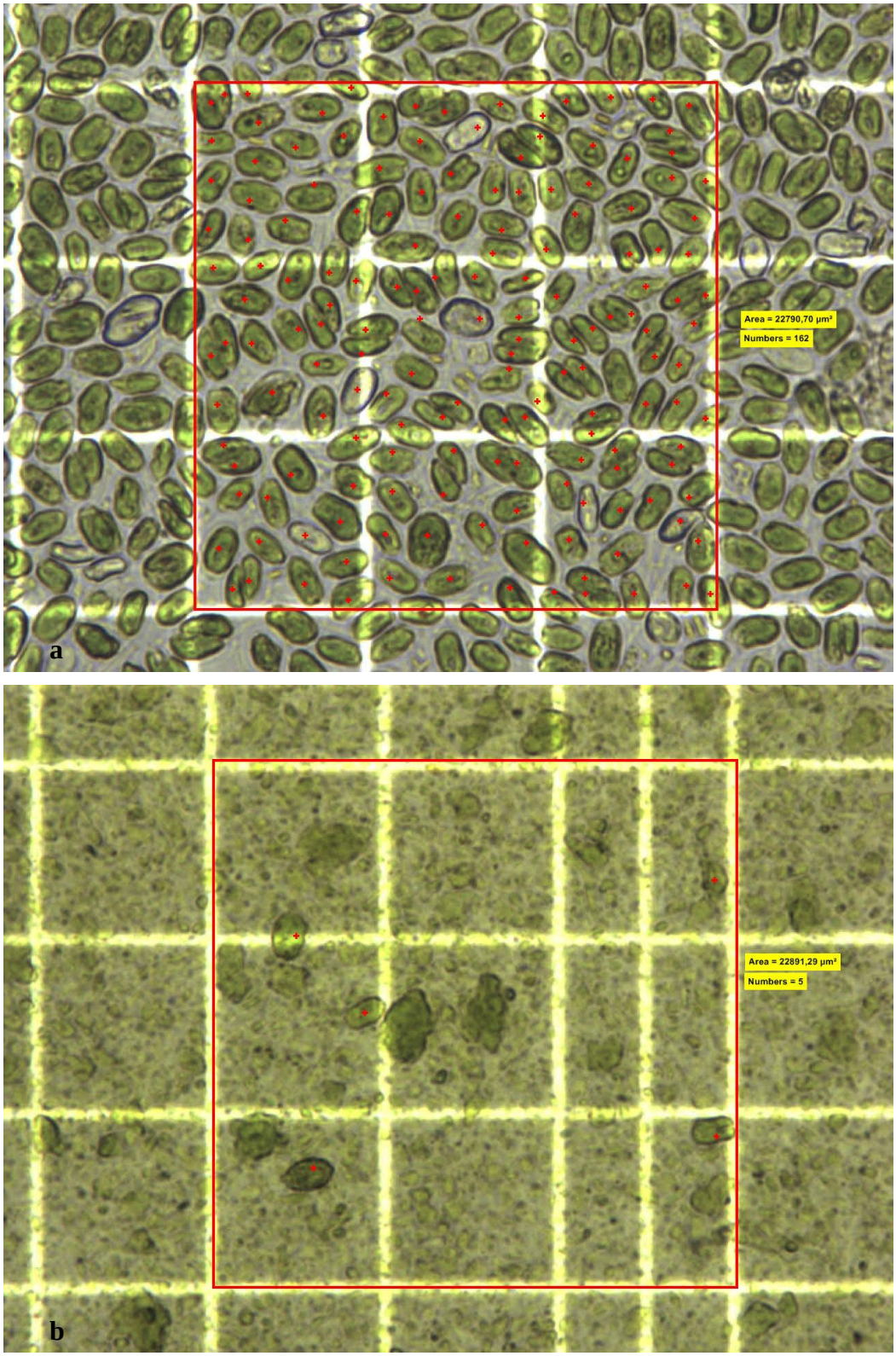

Figure 5: Neubauer squares as shown in the microscope, while counting. *T. chuii* (initial DM 22.5%) non-disrupted (a) and disrupted (b) cells.

2.3 Pulse Electric Fields (PEF) for enhancement of microalgae nutrients extraction

The PEF study was realized at the Laboratory of Food Chemistry and Toxicology of the Faculty of Pharmacy in the University of Valencia, Spain. Analytical methods for the determination of carotenoids, antioxidant activity and phenolic compounds in whole and processed biomass samples, were performed. Microalgae biomass was transferred by air from Norway to Spain, in insulating boxes with ice packs, and were stored at the university, in a freezer at -20^{o}C.

2.3.1 Microalgae preparation for PEF

Freeze dried biomass of *T. chuii* and frozen paste of *P. tricornutum* were used for PEF treatment. For the preparation of each sample, 198g of tap water were added to 2g biomass to end up with microalgae biomass solution of 1% DM according to Parniakov et al. (2014). When PEF is performed, salts are needed in the sample to conduct the electricity through the solution, thus tap and not deionized water was used for the preparation of the samples.

2.3.2 Pulse electric fields

For PEF treatment of the biomass, the PEF-Cellcrack III (German Institute of Food Technologies (DIL)) was used (Figure 6a). A chamber of 900mL capacity was chosen, the gap between the electrodes was set at 10 cm, and the mass added in the cell was always 200 gr. The specific energy input varied from 50 to 300 kJ/kg; the number of pulses from 23 to 1200 pulses, depending on the voltage applied (Table 8). Before and after treatment temperature and conductivity were measured in the sample, with a Portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics). From the 12

different runs carried out, Run 2 and 10 were chosen for further investigation of nutrient extraction, based on literature evaluation , and due to their relatively low specific energy (~100 KJ.kg).

Figure 6: PEF-Cellcrack III (DIL).

Table 8: Parameters and settings used for PEF treatment of microalgae biomass.

Run	Pulses	Voltage [kV]	Field strength [kV/cm]	Mass in cell [g]	Energy [J]/ pulse	Energy [J/kg]/ pulse	Specific energy [kJ/kg]
1	200	10	1	200	50	250	50
2	400	10	1	200	50	250	100
3	800	10	1	200	50	250	200
4	1200	10	1	200	50	250	300
5	50	20	2	200	200	1000	50
6	100	20	2	200	200	1000	100
7	200	20	2	200	200	1000	200
8	300	20	2	200	200	1000	300
9	23	30	3	200	450	2250	51.8
10	45	30	3	200	450	2250	101.3
11	89	30	3	200	450	2250	200.3
12	134	30	3	200	450	2250	301.5

2.3.3 Solvent extraction

After PEF treatment, a solvent was added in the samples 1:1 v/v with the scope to further enhance nutrient extraction. The solvents used were either Dimethyl Sulfoxide (DMSO) or distilled water (dH_2O). After solvent was added, the samples were stirred with rotating magnets at 400 rpm for either 4 or 24 hours at room temperature to test the effect of stirring time on compound extractability from the processed biomasses. After mixing, the samples were centrifuged for 10 min at 4000 rpm, using a 5810R Centrifuge (Eppendorf AG). The supernatant was collected and kept frozen at -20^{o}C for further analysis. Each sample was processed in each setting shown in Table 8 in duplicate.

2.4 Chemical characterization of processed microalgae biomass

Chemical analysis for the characterization of the processed biomass was carried out either at Nofima's accredited and research laboratories, Biolab (Bergen, Norway), and at the Laboratory of Food Chemistry and Toxicology of the Faculty of Pharmacy at the University of Valencia, Spain. The analysis carried out were crude (Biolab) and soluble protein (candidate), total lipids and fatty acids (Biolab), starch (Biolab), chlorophyll a and b (candidate), total polyphenols (candidate), total carotenoids (candidate), total antioxidant capacity (candidate), and microbiology (aerobic and anaerobic microorganisms, mold, yeast, *E.coli*) (Biolab). The analysis of chlorophyll a and b, total polyphenols, total carotenoids and total antioxidant capacity, were performed for training reasons and not to all the samples. The selected samples, with graded levels of cell wall disruption degree, from the bead milling trials (*P. tricornutum* and *T. chuii*

using small glass beads) are shown on Table 9. For confidentiality reasons, the analyses performed with Biolab's accredited methods are described only briefly.

For the analysis of, total polyphenols and total antioxidant capacity, extracts were prepared from 1g of microalgae sample, which was vortexed for 5 min with 9 mL of absolute ethanol (99.9% (Baker, Deventer, The Netherlands)). After vortexing, the samples were filtered (paper filter, pore size 10-20 um), and the extract was stored at -20^{o}C for further use. For the analysis of total carotenoids, chlorophyll a and b the same procedure was carried out, with the only difference being the use of methanol (95% (Baker, Deventer, The Netherlands)) instead of ethanol.

Table 9: Bead milling setup and disruption degrees of *T. chuii* and *P. tricornutum* samples used for learning analytical methods (chlorophyll a;b, total polyphenols, total carotenoids and total antioxidant capacity).

***T. chuii* disrupted by bead milling with small (0.2-0.4) glass beads**			
Disruption	**Dry matter %**	**Flow rate kg/h**	**Tip speed m/sec**
0%	-	-	-
61%	10	9	9
67%	10	11	9
77%	15	12	8
83%	18	9	10
90%	18	10	8
96%	18	6	11
99%	23	4	12
***P. tricornutum* disrupted by bead milling with small (0.2-0.4) glass beads**			
Disruption	**Dry matter %**	**Flow rate kg/h**	**Tip speed m/sec**
0%	-	-	-
48%	15	8	9
55%	18	12	12
61%	18	12	8
65%	15	12	11
72%	18	7	10
73%	21	8	11

2.4.1 Crude and soluble protein

Dried microalgae biomass samples were analyzed for crude protein: (Kjeldahl method N x 6.25; ISO 5983-1997). For soluble protein determination, the colorimetric method of Bicinchoninic Acid Assay (BCA assay) was used. Dried sample extracts were prepared as follows: Stock solutions were prepared with 500 mL of ultrapure water and the addition of NaOH powder, to adjust the solution's pH at 12.1g of freeze-dried sample (Table 9) was added to 50 mL of stock solution; then heated to 40^{o}C with continuous stirring for 1h. Centrifugation at 20 000 g for 20 min was followed, and the supernatant was collected for determination of the (solubilized) protein content. Fresh sample extracts were prepared as follows: After diluting the microalgae biomass with distillated water to reach a final DM of 0.875%, 20mL of the sample was centrifuged (Heraeus Multifuge X3R Centrifuge, Thermo Fisher Scientific) for 20 min at 20 000 g, and the supernatant was collected for determination of the (solubilized) protein content. In both cases, dried and fresh samples, the supernatant/extract was stored at -80^{o}C until further use.

Following, 2mL of microalgae extract were diluted two times with a lysis buffer (120 mM Tris, 4% SDS, pH 9), for prevention of soluble protein precipitation, and were then vortexed for 1 min. For the determination of protein in the algal solutions, the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific^{TM}) was used, which uses Albumin as standard. The Working Reagents (WR) and the Diluted Albumin (BSA) standards were prepared as described by the assay's supplier. Briefly, 0.1 mL of each standard and sample of unknown protein concentration were pipetted into a labeled 15mL Eppendorf tube (Figure 7). 2mL of the WR was then added, to each tube, and mixed well.

The tubes were covered and incubated for 30min at 37ºC. Following, the tubes were cooled to room temperature. A spectrophotometer (Thermo Scientific™ Evolution™ 201/220 UV-Visible Spectrophotometer) was used to measure the absorbance of all samples, over 10 min at 562nm wavelength. All analyses were performed in duplicate. If differences between parallels exceeded 5%, new duplicate analyses were carried out.

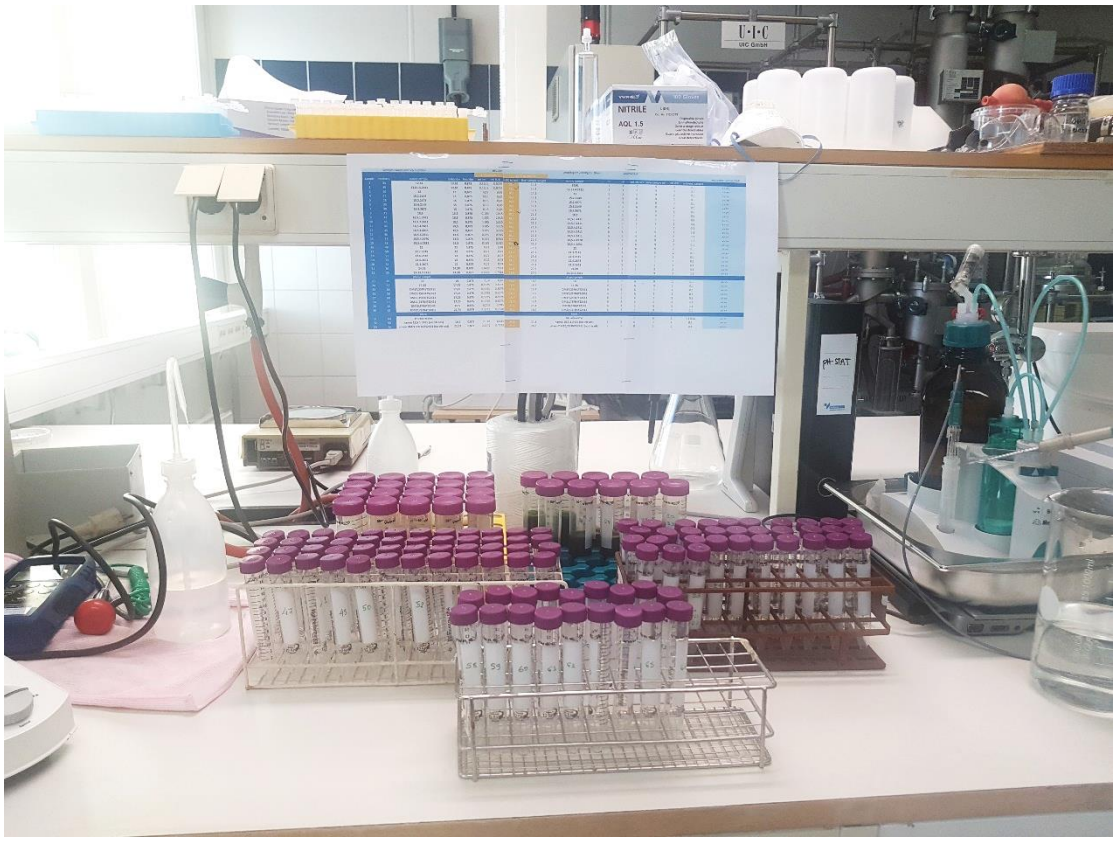

Figure 7: Preparation of the microalgae sample for BCA analysis.

2.4.2 Total lipids and fatty acids

Analysis of fatty acid composition was realized in Bligh & Dyer extracts (Bligh and Dyer 1959). Preparation of fatty acid methyl esters (FAME) was done according to AOCS Official Method Ce 1b-89. The Gas Chromatography (GC) analyses was conducted on a Trace GC gas chromatograph (Thermo Fisher Scientific) with a flame ionization detector (GC–FID), equipped with a 60 m × 0·25 mm BPX-70 cyanopropyl column with 0.25 μm film thickness (SGE Analytical Science). Helium was used as the mobile phase under the pressure of 60 bar. The injector temperature was 250 °C and the detector temperature was 260 °C. The oven was programmed as follows: 60 °C for 4 min, 30 °C/min to 164 °C, and then 1·0 °C/min to 213 °C, and 100 °C/min to 250 °C where the temperature was held for 10 min. The FAME were identified by comparing the elution pattern and relative retention time with the reference FAME mixture (GLC-793; Nu-Chek Prep Inc.). Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-793 mixture. Fatty acid composition was calculated using 23:0 FAME as the internal standard and reported on a sample basis as g/100 g FAME. All analyses were performed in duplicate. If differences between parallels exceeded Biolabs' standardized values, new duplicate analyses were carried out according to accredited procedures.

2.4.3 Trolox equivalent antioxidant capacity (TEAC)

For antioxidant capacity determination, Trolox equivalent was used. The value of TEAC (millimolar Trolox equivalents, mMTE) measures the antioxidant capacity of a given substance, as compared to the standard, Trolox (Sigma-Aldrich, Steinheim, Germany). TEAC was measured using the method (Re *et al.*, 1999) based on application of ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) Decolorization Assay (Sigma-Aldrich, Steinheim, Germany).

ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS 7mM stock solution with 140 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The solution was then diluted with ethanol (Baker, Deventer, The Netherlands) until the absorbance of 0.70 AU was reached at 734 nm. Once the necessary absorbance was reached, 2 mL of $ABTS^{+}$ was mixed with 100 µl of extract and the sample was incubated for 20 min at 20 °C. The absorbance was measured at the wavelength of 734 nm (Perkin Elmer Lambda2s spectrophotometer). All analyses were performed in triplicate. If differences between parallels exceeded 5%, new duplicate analyses were carried out.

2.4.4 Total phenolic compounds (TPC)

For determination of total polyphenols, (mg of gallic acid equivalent/L of extract, mgGAE/L) the Folin–Ciocalteu method was used, that is based on colorimetric oxidation/reduction reaction of phenols (Singleton, Orthofer, and Lamuela-Raventos, 1999). The method uses Gallic acid (Sigma-Aldrich, France) as standard. First, 50% v/v Folin–Ciocalteu reagent (Sigma–Aldrich, France), 2% sodium carbonate (Na_2CO_3) (VWR, France), as well as the diluted Gallic acid standards were prepared. Then, 100 µl

of sample extract was mixed with 3 mL of Na_2CO_3, and finally 100 µL of Folin-Ciocalteu reagent were added to this mixture. The samples were incubated for 1 h at room temperature. The absorbance was measured at the wavelength of 750 nm (Perkin Elmer Lambda2s spectrophotometer). All analyses were performed in triplicate. If differences between parallels exceeded 5%, new duplicate analyses were carried out.

2.4.5 Total carotenoids

Carotenoid content (C_{x+c}) was estimated spectrophotometrically according to the method of Lichtenthaler and Buschmann (2001). Aliquots of the extracts were diluted 15-300 times with 90 % (v/v) methanol in water and absorbances (A) were measured at 470 (A_{470}), 652.4 ($A_{652.4}$), and 665.2 ($A_{665.2}$) nm wavelength. Carotenoid content (C_{x+c}) was calculated using the Lichtenthaler equations (Eq.2). Chlorophyll A (C_a) and B (C_b) were also determined by the use of the Lichtenthaler equations (Eq.3 and Eq. 4). All analyses were performed in triplicate. If differences between parallels exceeded 5%, new duplicate analyses were carried out.

Eq. 2 $$C_a\left(\frac{\mu g}{ml}\right) = 16.82\ A_{665.2} - 9{,}28\ A_{652.4}$$

Eq. 3 $$C_b\left(\frac{\mu g}{ml}\right) = 36.92\ A_{652.4} - 16{,}54\ A_{665.2}$$

Eq. 4 $$C_{x+c}\left(\frac{\mu g}{ml}\right) = (1000A_{470} - 1.91\ C_a - 95.15\ C_b)/225$$

2.4.6 Starch

The starch content of freeze-dried *T. chuii* (Table 9) was determined by enzymatic degradation of starch to glucose with α-amylase and amyloglucosidase, using the total starch assay procedure from Megazyme (Megazyme 2009) accepted by AOAC — Association of Analytical Communities (Official Method 996.11) and AACC — American Association of Cereal Chemists (Method 76.13). The enzymatic method for resistant starch proposed by Megazyme (Megazyme 2009) was also performed. Resistant starch is passing through digestive tract unchanged (resistant to digestion) and is supposed to lower blood sugar levels naturally (Lockyer & Nugent, 2017).

2.4.7 Count of: aerobic microorganisms, mold and *E.coli*

Aerobic microorganisms, *E.coli* and mold were counted (Figure 8) at Biolab, using accredited methods. A Petrifilm™ Aerobic Count (3M, St. Paul, Minnesota, USA) was used, following the AFAQ/AFNOR 3M 01/1-09/89 method (AFAQ and AFNOR, 2010a). 3M E.coli/ Coliform plates were also used to determine *E. coli* , in which typical colonies were counted (NordVal validated method 3M 014-11). Petrifilm™ Rapid Yeast and Mold (RYM) count plates were used to determine mold content by AFNOR 3M 01/13-07/14.

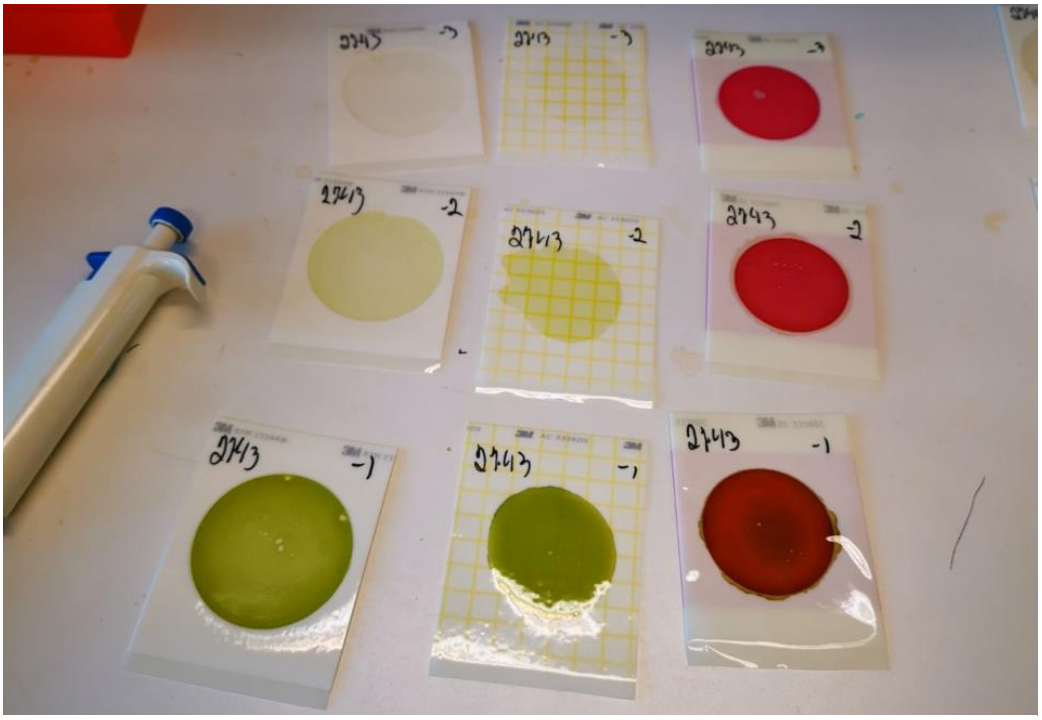

Figure 8: Count plates, for the determination of aerobic microorganisms, *E.coli* and mold on *T. chuii* samples.

2.5 Scanning Electron Microscopy (SEM) observation of microalgae cells

Freeze dried samples of whole and disrupted *P. tricornutum* and *T. chuii* were prepared and observed by scanning electron microscopy at the Department of Ichthyology and Aquatic Environment of the University of Thessaly, in Greece. For sample preparation, small pieces of the dried algae conglomerates were taken with the use of soft forceps and placed on double sided conductive tape. Following that, the samples were covered with a thin layer of gold using a sputter coater (Bal-tec SCD 004), under Argon (Ar) gas for 120 sec at 40mA. A scanning electron microscope (Cambridge Stereoscan 240) was used for observation of the specimens.

2.6 Viscosity determination of microalgae biomass

Viscosity of microalgae biomasses was measured using a RheolabQC rheometer (Anton Paar GmbH, Graz, Austria) according to a method developed by Biolab, Nofima (SSF reports D409 and B412. SSF1, 1987). The rheometer measures the viscosity of the sample by measurement of rotational torque and speed at specific temperature (25^{o}C).

2.7 Cell wall disruption experimental designs

2.7.1 Central Composite Design

Evaluation and optimization of the cell wall disruption of *P. tricornutum* using bead milling and either glass or Zr beads was carried out by use of three-factor central composite designs (CCD), comprising 17 settings, including 6 axial points (α) and 3 central points (Table 10). The distance from the axial points to the center points was calculated by the equation $\alpha = (2^{k})^{1/4}$, where k is the number of independent variables. The design included three independent variables: microalgae biomass DM, flow rate (as a measure of retention time of the biomass in the milling chamber) and agitator tip speed. The main response variable was % disruption efficiency In the design using glass beads, EPA+DHA release was also measured; whereas in the design where Zr beads were used, % Aerobic bacteria reduction, % Soluble protein release, % Fat release and biomass viscosity, were also measured in the differentially disrupted samples. The raw data from the above designs are presented in Appendix 1. The experimental data were analyzed by a second order polynomial equation (Eq. (5)):

$$Eq.\ 5 \qquad y = \beta_0 + \sum_{i=j}^{3} \beta_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j + \sum_{i=j}^{3} \beta_{ii} x_i^2 + \varepsilon$$

Where y is the predicted response, β_0 is the intercept, βi, βij and βii are the measurements of the effects of variables x_i, $x_i x_j$ and x_i^2 respectively. Where x_i represents linear coefficient, the $x_i x_j$ represents the first order interactions between x_i and x_j ($i < j$), x_i^2 quadratic coefficient and ε is the residual (error). The best fitted regressors in the model were identified by use of backward elimination of insignificant (P remove>0.05) variables using the Statistica Programme for Windows. The results obtained from the experiment were submitted to analyses of variance (ANOVA). R^2 values and F-test were used to evaluate the quality of the models. Outliers were detected based on normal probability plot of Studentized residuals and removed before final modelling of the respective responses.

2.7.2 Full factorial designs

Evaluation and optimization of the cell wall disruption of *T. chuii* by bead milling was performed with a full factorial, mixed level, design. Three independent variables were used: dry matter (DM) , flow rate (FR) and tip speed (TS), with mixed levels, and a response variable: % disruption efficiency. DM and TS were tested in five

levels; whereas biomass FR was tested in three levels (Table 11) with one replication. Raw data of the above design are presented in Appendix 2.

Evaluation of electropermeabilization of *T. chuii* and *P. tricornutum* biomass with pulsed electric fields (PEF) was performed using full factorial, mixed level, design. Three independent variables: treatment, extraction time, extraction solvent, with mixed levels; and five response variables: chlorophyll a, chlorophyll b, total carotenoids, total phenolic compounds (TPC) and trolox equivalent antioxidant capacity (TEAC). Extraction time and solvent were tested at two levels each (4 *vs* 24h & water *vs* DMSO, respectively), whereas treatment was a three-level factor (No treatment, PEF1, PEF2).

Table 10: Central Composite design **(L_{17}) used for bead milling of *P. tricornutum* biomass with glass or Zr beads; with 3 variables:** (X_1:Biomass Dry matter in %, X_2: Measured biomass flow rate in kg/g, X_3: Tip speed in m/sec).

Run	X_1 Dry matter	X_2 Flow rate	X_3 Tip Speed	
1	-1	-1	-1	**Cube point**
2	1	-1	-1	**Cube point**
3	-1	1	-1	**Cube point**
4	1	1	-1	**Cube point**
5	-1	-1	1	**Cube point**
6	1	-1	1	**Cube point**
7	-1	1	1	**Cube point**
8	1	1	1	**Cube point**
9	-1.682	0	0	**Star point**
10	1.682	0	0	**Star point**
11	0	-1.682	0	**Star point**
12	0	1.682	0	**Star point**
13	0	0	-1.682	**Star point**
14	0	0	1.682	**Star point**
15	0	0	0	**Centre point**
16	0	0	0	**Centre point**
17	0	0	0	**Centre point**

Raw data of the above design are presented in Appendix 3. All processing points in the design were realized in two replicates. The results obtained from the experiment were submitted to analyses of variance (ANOVA).

2.5.4 Statistical analysis

Raw data were treated in Microsoft Excel 2013 (Microsoft Corp., Redmond, WA), and statistical analyses were performed with STATISTICA (v.12.0) from Statsoft (Tulsa, OK, USA) and IBM SPSS Statistics for Windows (v. 25.0) from Armonk, NY: IBM Corp. When ANOVA analysis were carried out, data were checked for homogeneity of variance. Duncan's Multiple Range post hoc test (DMRT) was used to rank significantly different response mean values ($P<0.05$) for variables with 3 or more levels.

Table 11: Full factorial, mixed level, design (L_{75}) used for bead milling of *T. chuii* biomass with 3 variables: (X_1: DM biomass DM in % and 5 levels, X_2: Measured biomass flow rate in kg/g and 3 levels of pump setting, X_3: Tip speed in m/sec and 5 levels).

Run	X_1	X_2	X_3	Run	X_1	X_2	X_3
	DM %	Flow rate kg/h	Tip speed (m/sec)		DM %	Flow rate (kg/h)	Tip speed (m/sec)
1	1	1	1	**39**	3	2	4
2	1	1	2	**40**	3	2	5
3	1	1	3	**41**	3	3	1
4	1	1	4	**42**	3	3	2
5	1	1	5	**43**	3	3	3
6	1	2	1	**44**	3	3	4
7	1	2	2	**45**	3	3	5
8	1	2	3	**46**	4	1	1
9	1	2	4	**47**	4	1	2
10	1	2	5	**48**	4	1	3
11	1	3	1	**49**	4	1	4
12	1	3	2	**50**	4	1	5
13	1	3	3	**51**	4	2	1
14	1	3	4	**52**	4	2	2
15	1	3	5	**53**	4	2	3
16	2	1	1	**54**	4	2	4
17	2	1	2	**55**	4	2	5
18	2	1	3	**56**	4	3	1
19	2	1	4	**57**	4	3	2
20	2	1	5	**58**	4	3	3
21	2	2	1	**59**	4	3	4
22	2	2	2	**60**	4	3	5
23	2	2	3	**61**	5	1	1
24	2	2	4	**62**	5	1	2
25	2	2	5	**63**	5	1	3
26	2	3	1	**64**	5	1	4
27	2	3	2	**65**	5	1	5
28	2	3	3	**66**	5	2	1
29	2	3	4	**67**	5	2	2
30	2	3	5	**68**	5	2	3
31	3	1	1	**69**	5	2	4
32	3	1	2	**70**	5	2	5
33	3	1	3	**71**	5	3	1
34	3	1	4	**72**	5	3	2
35	3	1	5	**73**	5	3	3
36	3	2	1	**74**	5	3	4
37	3	2	2	**75**	5	3	5
38	3	2	3				

Table 12: Full factorial, mixed level, designs (2 x L_{24x2}) with 2 replications of each design point, used for PEF treatment of *T. chuii* and *P. tricornutum* biomasses, with 3 variables: *(X1: Solvent type in 2 levels; water and DMSO, X2: Mixing time in 2 levels 4 and 24 h, and X3: Pre-treatment in 3 levels; none, PEF1 and PEF2.*

Run	Species	X_1	X_2	X_3		Run	Species	X_1	X_2	X_3
		Solvent	Time (h)	Pre-Treatment				Solvent	Time (h)	Pre-Treatment
1	*T. chuii*	1	1	1		1	*P. tricornutum*	1	1	1
2	*T. chuii*	1	1	2		2	*P. tricornutum*	1	1	2
3	*T. chuii*	1	1	3		3	*P. tricornutum*	1	1	3
4	*T. chuii*	1	1	1		4	*P. tricornutum*	1	1	1
5	*T. chuii*	1	1	2		5	*P. tricornutum*	1	1	2
6	*T. chuii*	1	1	3		6	*P. tricornutum*	1	1	3
7	*T. chuii*	1	2	1		7	*P. tricornutum*	1	2	1
8	*T. chuii*	1	2	2		8	*P. tricornutum*	1	2	2
9	*T. chuii*	1	2	3		9	*P. tricornutum*	1	2	3
10	*T. chuii*	1	2	1		10	*P. tricornutum*	1	2	1
11	*T. chuii*	1	2	2		11	*P. tricornutum*	1	2	2
12	*T. chuii*	1	2	3		12	*P. tricornutum*	1	2	3
13	*T. chuii*	2	1	1		13	*P. tricornutum*	2	1	1
14	*T. chuii*	2	1	2		14	*P. tricornutum*	2	1	2
15	*T. chuii*	2	1	3		15	*P. tricornutum*	2	1	3
16	*T. chuii*	2	1	1		16	*P. tricornutum*	2	1	1
17	*T. chuii*	2	1	2		17	*P. tricornutum*	2	1	2
18	*T. chuii*	2	1	3		18	*P. tricornutum*	2	1	3
19	*T. chuii*	2	2	1		19	*P. tricornutum*	2	2	1
20	*T. chuii*	2	2	2		20	*P. tricornutum*	2	2	2
21	*T. chuii*	2	2	3		21	*P. tricornutum*	2	2	3
22	*T. chuii*	2	2	1		22	*P. tricornutum*	2	2	1
23	*T. chuii*	2	2	2		23	*P. tricornutum*	2	2	2
24	*T. chuii*	2	2	3		24	*P. tricornutum*	2	2	3

3.0 Results and Discussion

3.1 Cell wall disruption by bead milling for release of nutrients

3.1.1 *Tetraselmis chuii*

Our model showed significant correlation between disruption efficiency and bead mill parameters ($P < 0.000$). Normal probability plot of the residuals showed a normal distribution between the predicted and observed results, apart from two outliers which were removed before final modelling of the respective responses (Run 7 and 12 from Table 11) (Appendix 4). Based on response surface regression analysis with backward removal (Appendix 5), cell wall disruption efficiency of *T. chuii* with small glass beads was affected by all tested variables, i.e. dry matter (DM), flow rate (FR), (Figure 10 and 12) and the interaction of flow rate with tip speed (TS) (Figure 11). The response model (R^2 =0.82) shows a positive DM effect on the disruption efficiency. A negative FR^2 effect and a positive TS×FR effect is also observed (Table 9). The analysis of variance showed no lack of fit. All models showed that DM and TS have a positive effect on % Disruption efficiency; while the squared FR affected it negatively, creating curvature in the model response surface.

Table 9: Regression coefficients and significance (p) values after backward elimination.

	Effect										
	Intersept	DM	DM^2	FR	FR^2	TS	TS^2	DM×FR	DM×TS	FR×TS	R^2
Disruption	66.625*	1.2288*	ns	ns	-0.084*	ns	ns	ns	ns	0.11**	0.821094
											* p<0.00000
											** p<0.00002

Accordingly we performed a meta-analysis, using general regression models (GRM), of data from Doucha et al. (2008) on *Chlorella vulgaris* cell wall disruption efficiency using bead milling, and could observe that the effects of biomass DM, FR and bead size on cell wall disruption are statistically significant, with significant interaction between DM and FR as well as DM and bead size, but not that of TS, though this was not discussed in the paper.

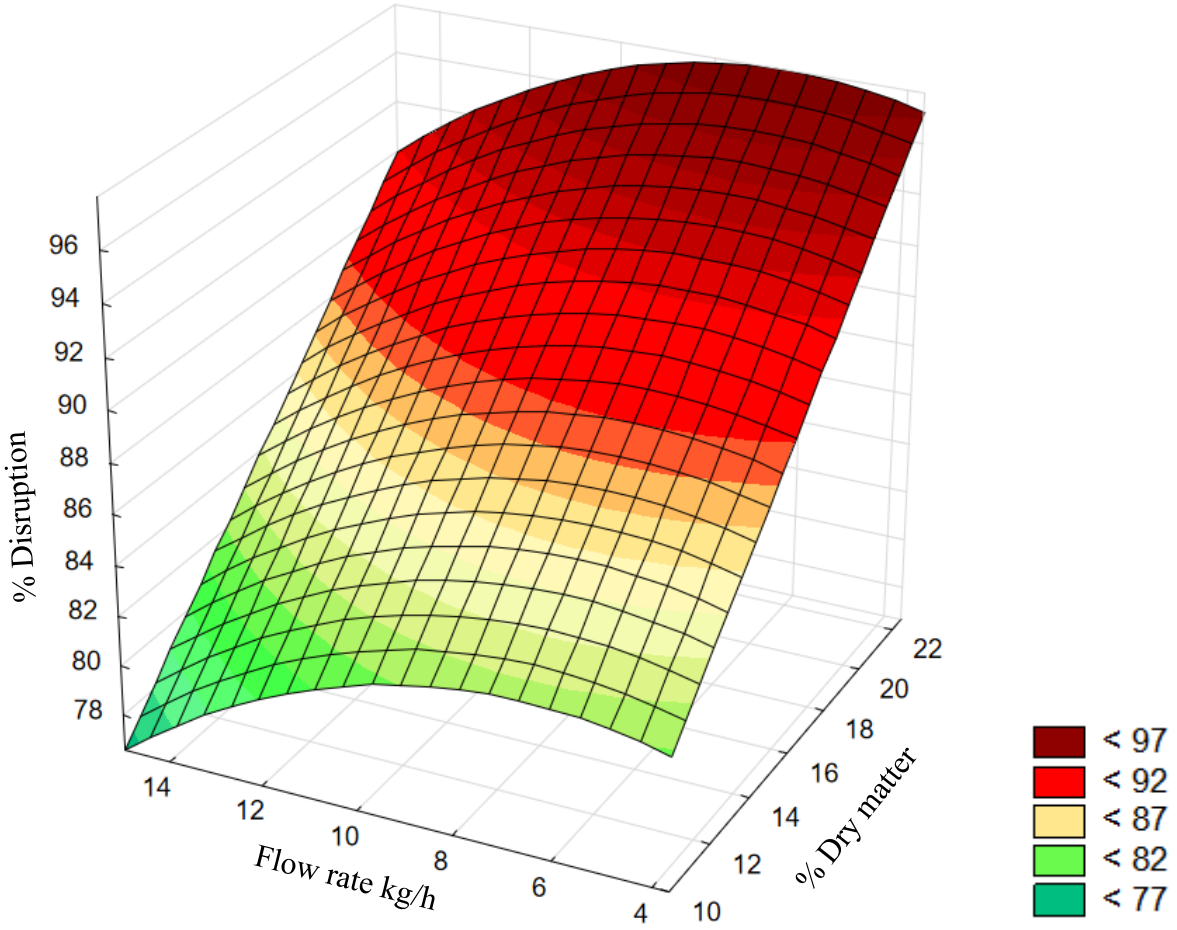

Figure 10: Disruption efficiency of *T. chuii* by bead milling with small glass beads. Effect of flow rate (kg/h) and % dry matter. The third variable (Tip speed) is set at the mean experimental value (10 m/sec).

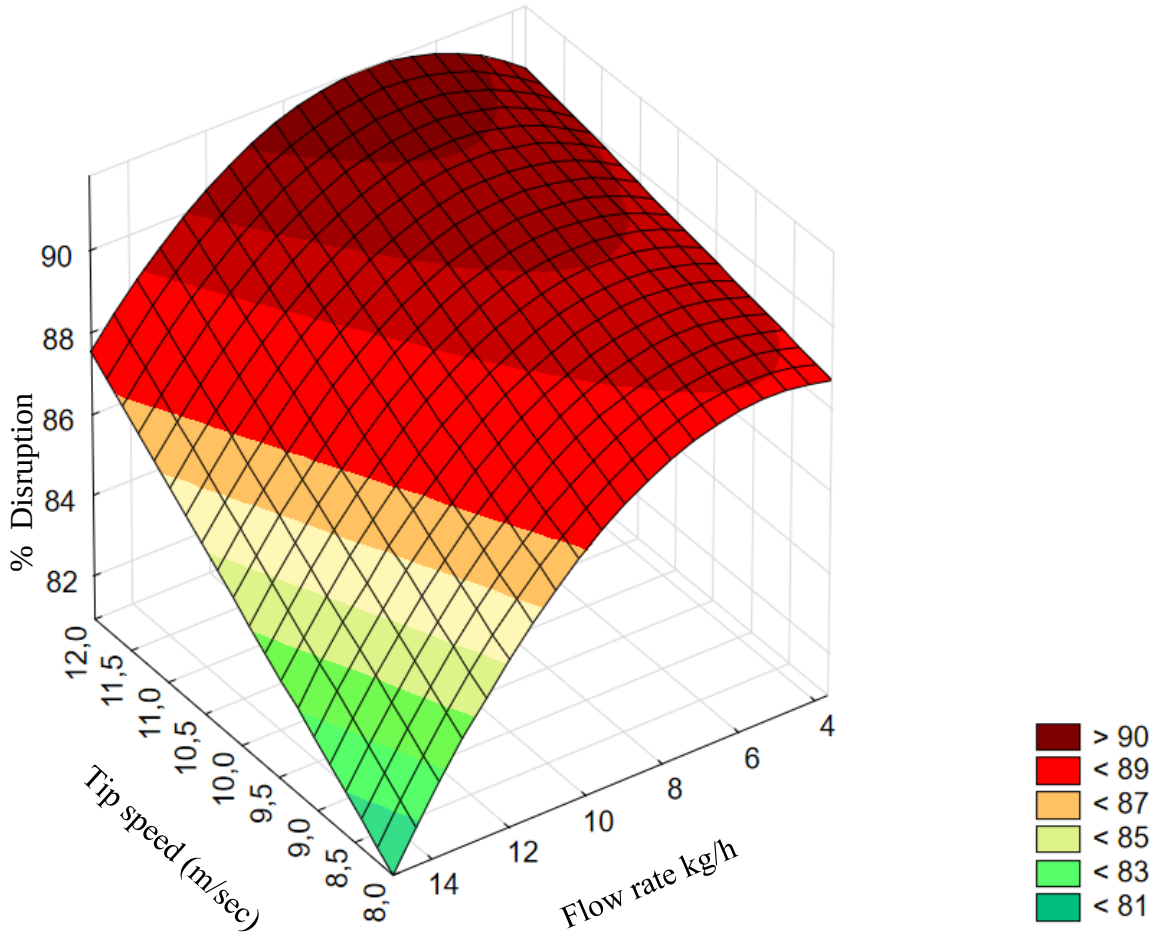

Figure 11: Disruption efficiency of *T. chuii* by bead milling with small glass beads. Effect of flow rate (kg/h) and tip speed (m/sec). The third variable (dry matter) set at the mean experimental value (16.25%).

As shown before, DM affects significantly the disruption efficiency of *T. chuii* biomass; the more concentrate is the algae biomass, the better disruption is achieved. This finding contradicts with other bead milling studies (Doucha and Lívanský, 2008; Postma *et al.*, 2017; Safi *et al.*, 2017) in which, low biomass concentration was chosen without prior investigation. Specifically, Postma *et al.* (2017) investigated the effect of bead milling on *T. suecica* biomass, with concentration approximately 9% DM. They achieved 99% disintegration after 6.6 minutes of continuous bead milling of recirculated

biomass. In our disintegration study, the maximum cell disruption of *T. chuii* biomass (i.e. >99%), was achieved under a single bead milling passage, and shorter retention time than in Postma et al., (2017) (~6.5 min) at higher biomass concentrations. Flow rate had a reverse effect on cell wall disintegration efficiency, with best results obtained at higher flow rates. However, the model showed curvature with deterioration of cell call disruption efficiency at flow rates below 7-8 kg/h, more prominent at higher tip speed rates (Figure 11).

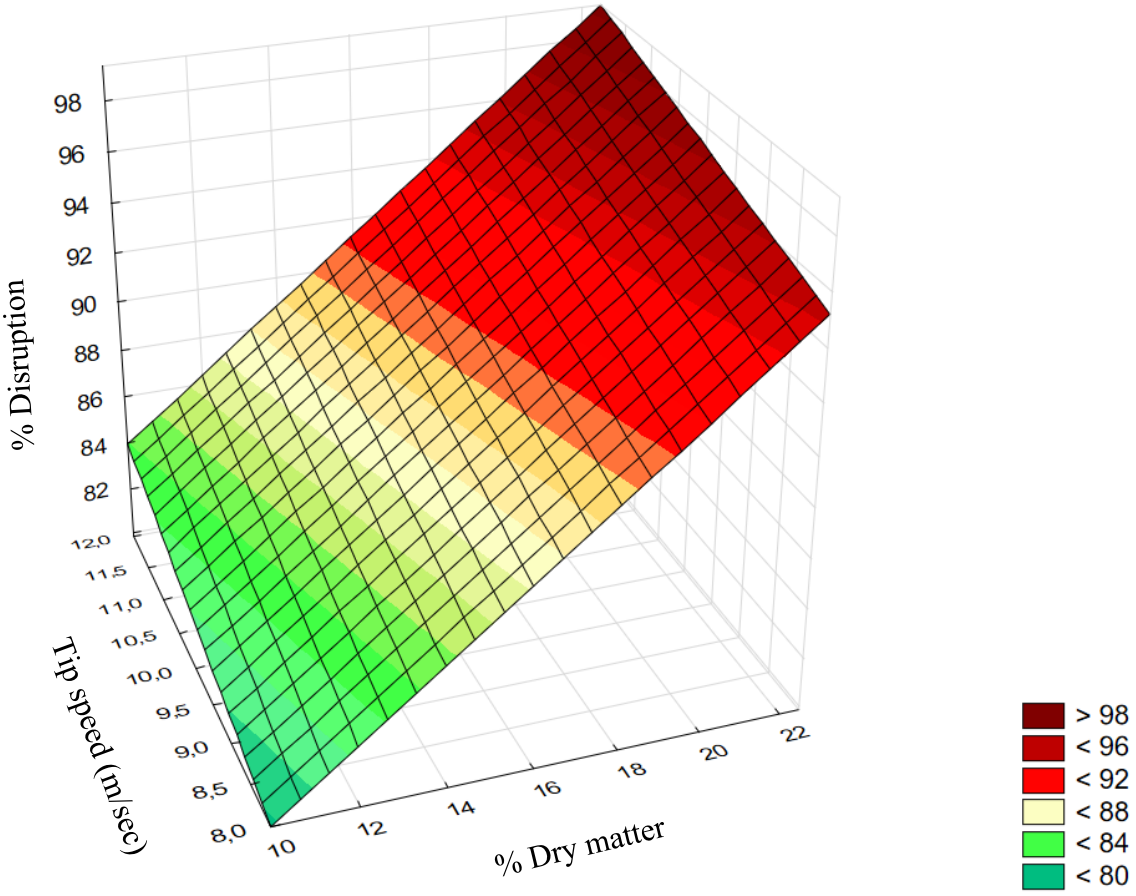

Figure 12: Disruption efficiency of *T. chuii* by bead milling with small glass beads. Effect of tip speed (m/sec) and % dry matter. The third variable (flow rate) set at the mean experimental value (9.3 kg/h).

The inversely proportional relation of disruption efficiency and flow rate increment has been also observed by Doucha and Lívanský (2008) and Montalescot et al. (2015). In both studies it was seen that disruption efficiency decreased with increasing flow rate, which is expected as retention time in the milling chamber decreases with increasing flow rate. Montalescot et al. (2015) for both studied microalgae species, *N. oculate* and *P. cruentum,* found that the optimal tip speed was the slowest (8 m/sec). In our study, increasing agitator tip speed led to weak but significant increase of disruption efficiency (Figure 12).

3.1.1a Nutrient extraction and disruption efficiency

Crude protein of *T. chuii* biomass was between 48% and 53% in DM and up to almost half of it was water soluble (Figure 13), which is similar as that analyzed in e.g. *Schizochytrium sp*. spray dried biomass (39.4% soluble protein of total crude protein) (Kousoulaki *et al.*, 2016).

Over 80% of the water-soluble protein was analyzed to be below 200 kDa (Figure 15), i.e. possibly composed of free amino acids, very small peptides and other small nitrogenous compounds. The water-soluble fraction of marine meals such as fish meal is known to have bioactive properties promoting a.o. feed intake, lipid utilisation and deposition and growth in farmed fish (Kousoulaki *et al.*, 2009; Kousoulaki *et al.*, 2013). Disruption efficiency of *T. chuii* biomass affected also the soluble protein extraction. Specifically, disrupted biomass had incresed soluble protein (% of total protein), compared to non disrupted (Figure 16) but higher soluble protein levels were not observed on higher disruption levels, as it would be expectet. This may be due to the

higher forces and energy levels involved in the processes resulting in higher levels of disruption which may have negatively affected the solubility of some proteins.

As for soluble protein, total antioxidant capacity (Figure 17), total phenolic compounds (Figure 18), as well as carotenoid content (Figure 19) of the whole and processed *T. chuii* biomass, did not follow the patterns of disruption. Nevertheless, higher compound levels were analyzed in all processed samples as compared to the whole biomass. Bunge et al. (1992) described the so-called Stress Model (SM), which assumes that the disruption process in stirring mills, like bead mill, is regulated by chain reaction stress events and their intensity. Every energy change per unit of mass, is followed by a certain change of disruption efficiency no matter which operational parameter (i.e. bead type/size/feeling, flow rate, tip speed), influenced the energy input (Bunge *et al.*,1992). The same study emphasizes on the fact that a cell is either intact or disrupted and that from a disrupted cell, all intracellular components are assumed to be released, becoming bioavailable, which is apparently not always the case. SEM observations on freeze dried *T. chuii* cell disrupted with small glass beads (Figure 14) showed us cracked or completely broken cells. Such observations are not seen on an optic microscope. In our study, either the disruption efficiency was in some cases over or under-estimated (limited observation capacity of optic microscope), or the energy input during bead milling was so high, resulting in degradation of labile intracellular compounds.

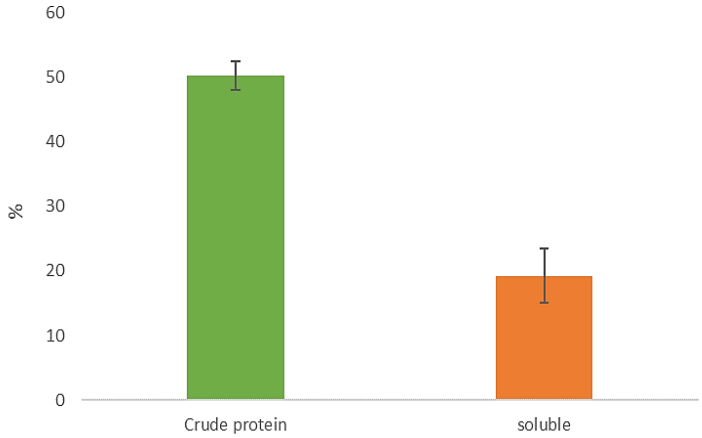

Figure 13: Crude and soluble protein of *T. chuii* biomass.

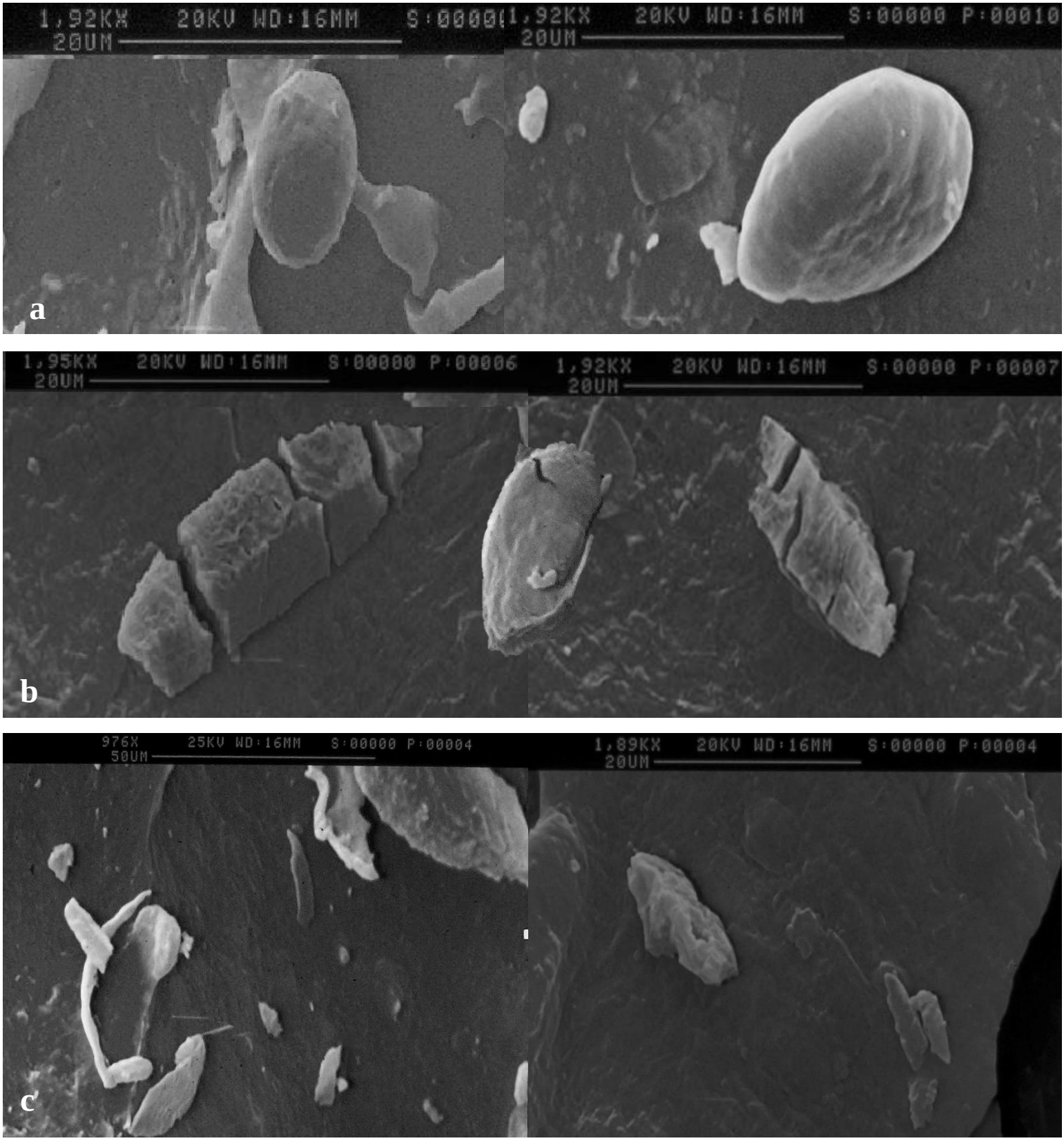

Figure 14: *T. chuii* cell observed under SEM. a) intact cells b) and c) disrupted by bead milling with small glass beads. *Scale bar on top of each picture set*

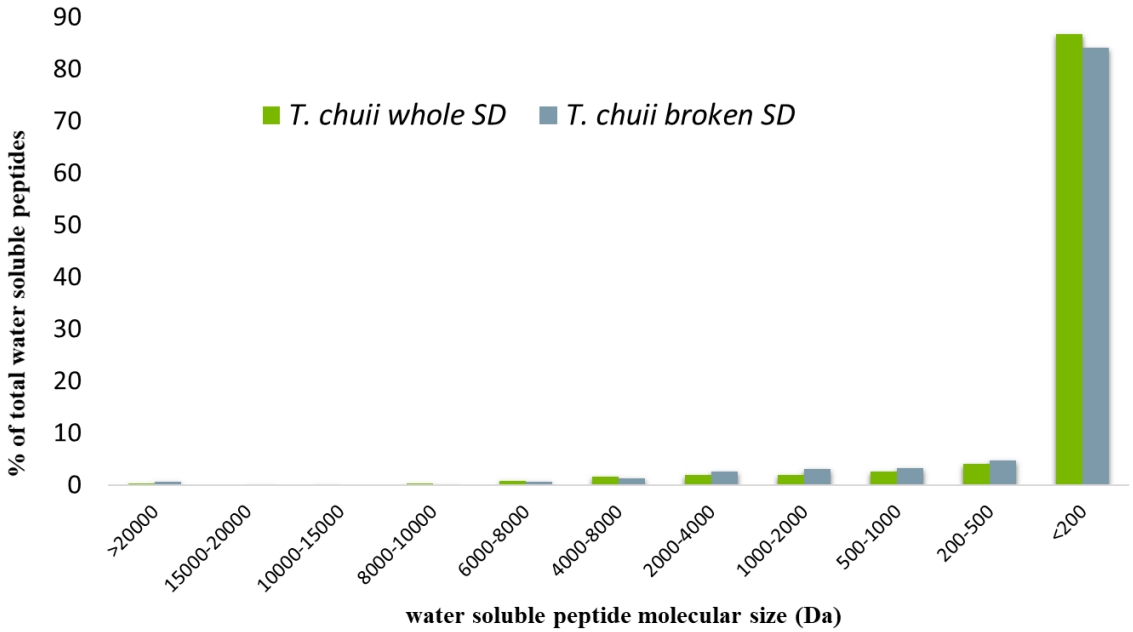

Figure 15: Water soluble peptide size distribution.
Whole and cell wall disrupted spray dried *T. chuii.*

Based on our results maximum soluble protein for *T. chuii* biomass, was achieved after 61% disruption; while 99% disruption had 5% decrease of the extracted soluble protein (Figure 17). Determination of total antioxidant capacity and total phenolic compounds revealed an unconventional pattern of the obtained results. For both analysis, 67% disruption gave the maximum result; 203.3 mg Gallic acid /g DW and 27.4 mM trolox/g DW respectively, followed by 99% and 61% disruption. Phenolic compounds have antioxidant activity, thus the correlation of the results obtained from the two methods may validate the observed patterns. Comparing our results with previous conducted studies on *Tetraselmis sp.* (as well as *T. chuii*), we conclude that more phenolic compounds were extracted even at the non-disrupted microalgae biomass. Namely, Widowati et al. (2017) extracted 16.87 mg GAE (Gallic acid Equivalent) g^{-1} from *T. chuii biomass* (50ppm concentration), whereas Maadane et al. (2015) 25.5±1.5 mg GAE g^{-1}.

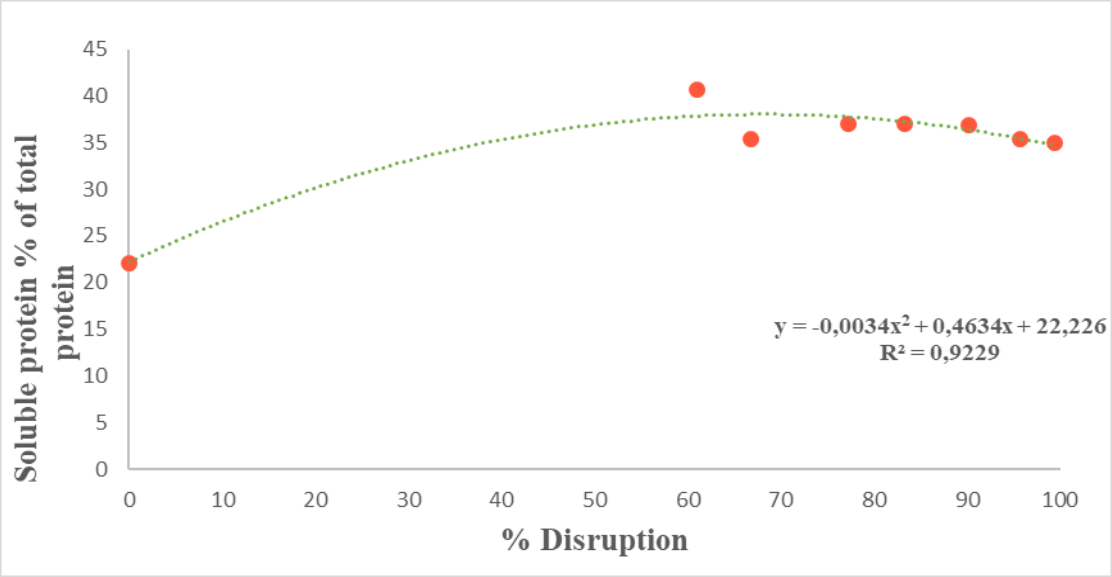

Figure 16: Correlation of cell wall disruption of *T. chuii* by bead milling with glass beads and analyzed soluble protein.

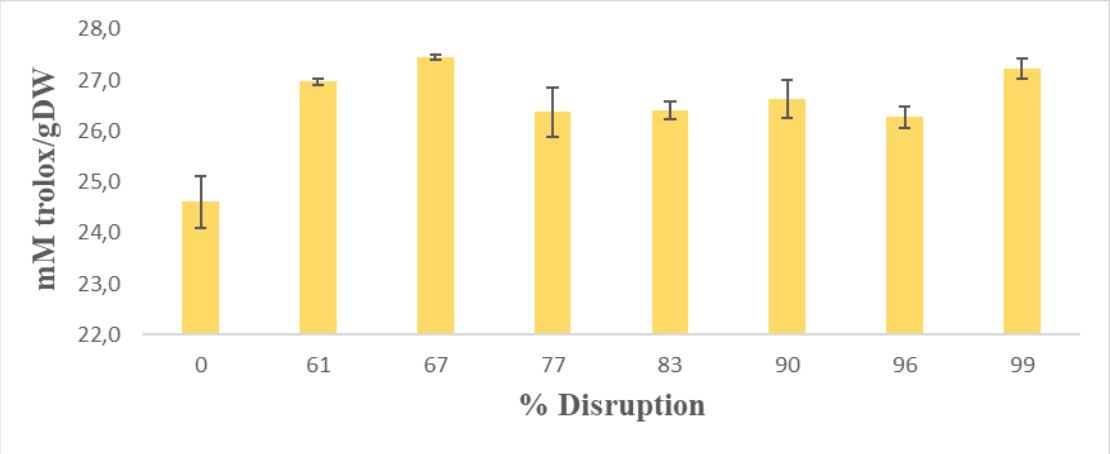

Figure 17: Total antioxidant capacity in *T. chuii* biomass samples.
Whole (0) or following bead milling with glass beads.

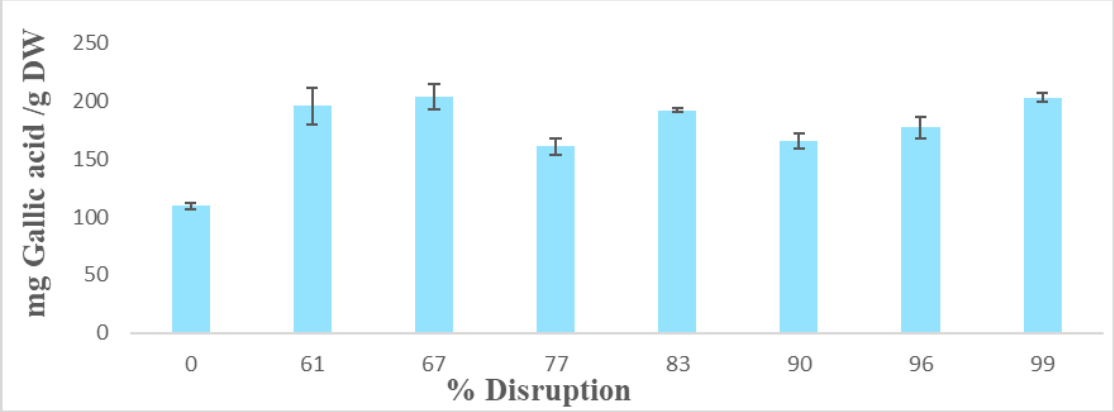

Figure 18: Total phenolic compounds in *T. chuii* biomass.
Whole (0) or following bead milling with glass beads.

Moreover, Goiris et al. (2012) extracted 3.74 ± 0.1 mg GAE g^{-1} DW from *Tetraselmis sp.* biomass, while from *T. suecica* only 1.71 ± 0.0.57 mg GAE g^{-1} DW. The aforementioned studies used the Folin-Ciocalteu procedure for phenolic content estimation, but none have disrupted microalgae cell wall prior extraction. In the same study, Maadane et al. (2015) extracted 17 times more carotenoids (total) from *T. suecica* biomass, and 11 times more from *Tetraselmis sp.*, than the levels extracted in our study (Figure 19). The difference between the results we obtained compared to the study of Maadane et al. (2015), may lie on the difference in the culture or analytical methods used or inherent differences in the two different species.

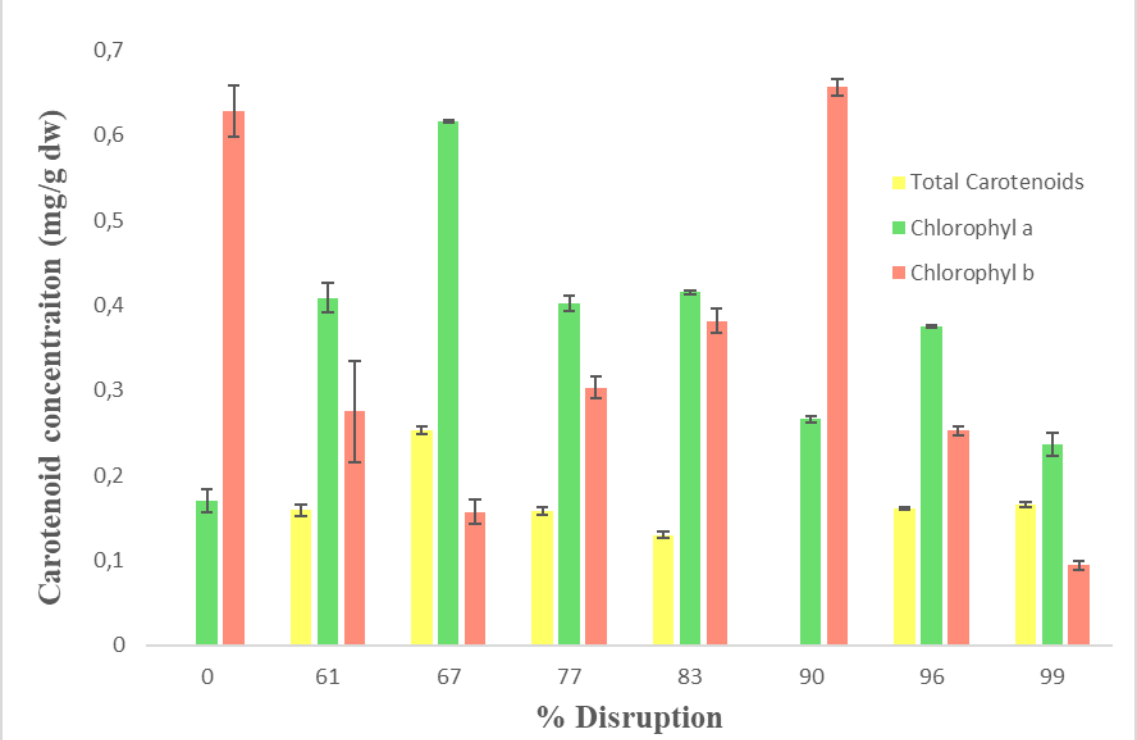

Figure 19: Determination of carotenoid content of *T. chuii* biomass.
Whole (0) or following bead milling with small glass beads.

The analyzed levels of starch in *T. chuii* biomass, was interestingly lower in the disrupted as compared to the whole biomass (Figure 20). Both starch and resistance starch were measured on selected *T. chuii* specimens with different degrees of disruption (from 0 to 99% disruption) showing the same pattern.

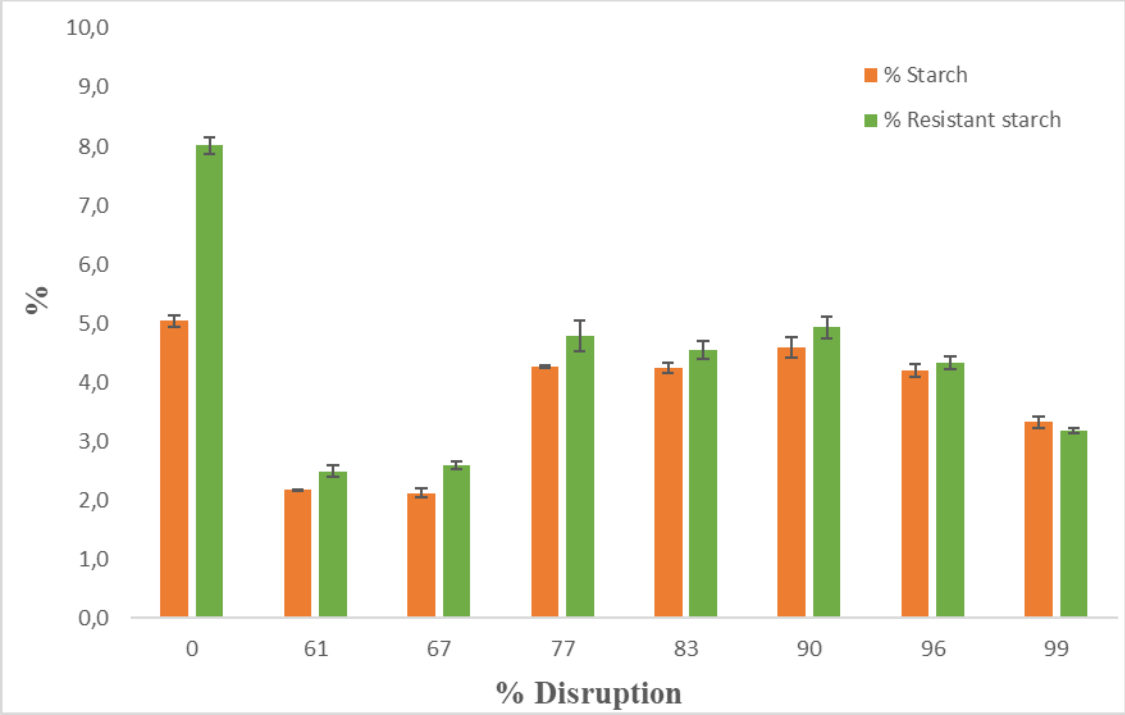

Figure 20: Determination of total starch content of *T. chuii* biomass.
Whole (0) or following bead milling with small glass beads.

The accumulation of microalgal starch is related to species and cultivation-environmental conditions (Yao *et al.*,2013); whereas starch accumulation is enhanced by macro element limitation (Dragone *et al.*, 2011). In their study, Dragone et al. (2011), concluded that *Chlorella sp.* under certain nutrient limitations could reach 8-fold higher starch accumulation (i.e. 41% of dry cell weight). *T. chuii* starch levels under normal growth conditions range from 2% to 10% (in dry weight), whereas after stress, starch levels can increase up to 20-42% (in dry weight) (personal communication with Dr. Dorinde Kleingris, NORCE/National Algae pilot Mongstad - NAM). In our case, *T. chuii*

biomass, was produced under normal growing conditions, and starch enhanced accumulation was not expected; thus 5% starch dw^{-1} *T. chuii* (8% total starch (including resistance starch)) on unprocessed biomass is an expected value.

3.1.2 *Phaeodactylum tricornutum*

P. tricornutum disruption efficiency by bead milling, was examined based on central composite design. In our experiments, two different bead types were used; glass and zirconium (Zr). Beads size was kept constant at 0.25-0.4 mm diameter for glass beads, and 0.3mm diameter for Zr beads. The chamber volume used was 0.6 l, and the beads were added at 80% chamber filling rate. In both cases, normal probability plot of the residuals showed a normal distribution between the predicted and observed results (Appendix 6).

A significant correlation of disruption efficiency and bead mill parameters, Flow Rate (FR), Tip Speed (TS), and the interaction of the two, was observed (R^2= 0.645) on the disruption of *P. tricornutum* with small glass beads (Table 10) after backward elimination (Appendix 7). The response surface plot (Figure 21) has some weaknesses (Disruption is estimated up to 140%) us disruption cannot be above 100%. The expected

Table 10 : Regression coefficients and significance (p) values after backward elimination. Bead milling of *P.tricornutum* with small glass beads.

	Effect										
	Intersept	DM	DM^2	FR	FR^2	TS	TS^2	DM×FR	DM×TS	FR×TS	R^2
%Disruption	-160.14[a]	ns	ns	15.73[b]	0.26[c]	25.38[e]	ns	ns	ns	-2.17[d]	0.645
EPA+DHA release (g/100g Fat)	36.53**	-2.05*	0.06*	ns	ns	ns	ns	ns	ns	ns	0.703

* p<0.0001
** p<0.0000
[a] p=0.014
[b] p=0.005
[c] p=0.004
[d] p=0.002.
[e] p=0.001

curvature, which would have held the disruption into normal range, is not seen on the plot. The positive FR^2 in combination with the non-significance of TS^2 as long as the positive interaction of FR and TS (FRxTS), could have affected the accuracy of the model/plot. Thus, more experiments on the extreme values of DM and TS could give a better model. EPA+DHA release was only affected by the Dry Matter of the biomass (Table 10) and even though the model's coefficient of determination was strong (R^2= 0.703), visual representation of the model was not possible (Raw data of regression analysis with backward removal shown on Appendix 8). There was not a big variation between the maximum and minimum values of the released EPA+DHA (10.2 g and 11.2 g (/100g Fat)); thus, the non-significance of the bead milling parameters may lie on that limited variation.

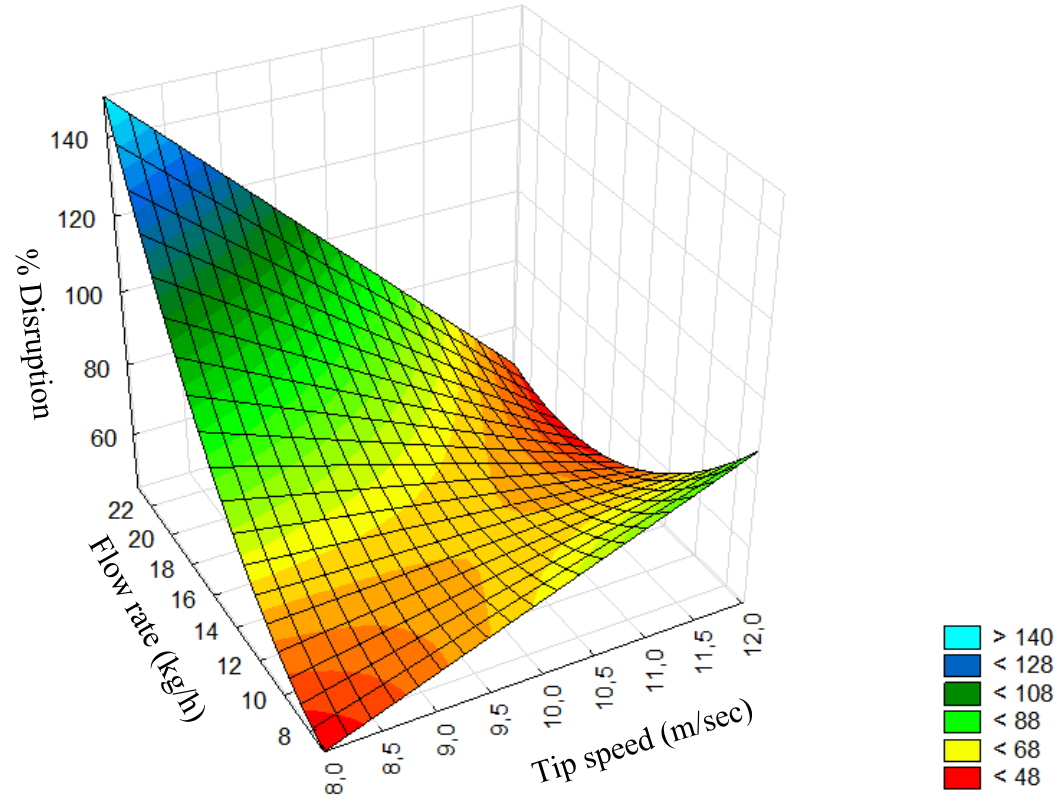

Figure 21: Disruption efficiency of *P. tricornutum* by bead milling with glass beads. Effect of flow rate (kg/h) and tip speed (m/sec).

P. tricornutum biomass, was disrupted more efficiently by bead milling with Zr beads. Almost 75% disruption was achieved by glass beads whereas 88% disruption was achieved by Zr beads. The response model (R^2= 0.99) (Raw data of regression analysis with backward removal shown in Appendix 9) showed significant correlation of bead milling parameters with disruption efficiency; namely, Dry matter, Flow rate, the interaction of the two (Figure 22), as well as the interaction of Dry matter and Tip speed (Figure 23). Comparing the two experiments, -beads milling with small glass beads/bead milling with small Zr beads- we can observe that the parameters affect differently the disruption of *P. tricornutum* cells. Specifically, even if FR affects both models, it affects positively the bead milling with glass beads experiment, while negatively the bead milling with Zr beads. Also, the interactions between DM, FR, and TS are not common in the two models; as bead milling with small glass beads is affected by FRxTS, whereas bead milling with small Zr beads is affected by DMxFR and DMxTS. These inconsistent correlations of bead milling parameters with disruption efficiency of the same microalgae species but with different bead type, are also described by Doucha and Livansky,2008 in their study on *Chlorella sp.* cells, with different bead types.

Table 11: Regression coefficients and significance (p) values after backward elimination.
Bead milling of *P.tricornutum* with small Zr beads.

	Effect										
	Intersept	DM	DM^2	FR	FR^2	TS	TS^2	DM×FR	DM×TS	FR×TS	R^2
Disruption	75.52^{***}	ns	-0.65^{***}	-107.28^{***}	ns	ns	-22.81^{***}	0.57^{***}	24.38^{***}	ns	0.99
Aerobic bacteria	73.46^{***}	ns	ns	5.88^{*}	ns	ns	ns	-0.44^{***}	ns	ns	0.815
Viscosity/10 (mPa)	-77.85^{a}	ns	ns	ns	0.44^{b}	ns	ns	ns	2.69^{***}	-2.35^{c}	0.874
% Soluble protein release	59.28^{***}	ns	ns	ns	ns	ns	ns	ns	ns	ns	0
% Fat release	15.13^{***}	ns	ns	0.76^{**}	ns	ns	ns	-0.04^{**}	ns	ns	0.756

* p<0.0001
** p<0.00001
***p<0.00000
[a] p=0.06
[b] p=0.005
[c] p=0.0007

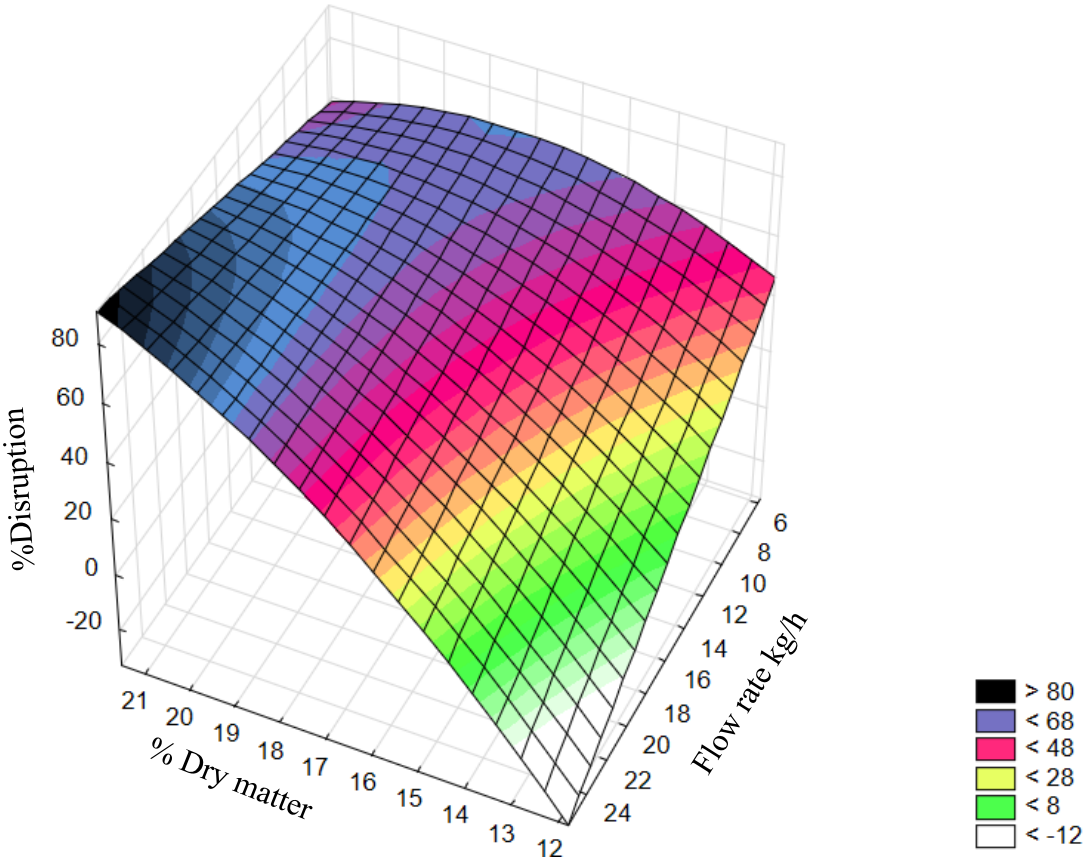

Figure 22: Disruption efficiency of *P. tricornutum* by bead milling with Zr beads. Effect of flow rate (kg/h) and dry matter (%). The third variable (Tip speed) is set at the mean experimental value (8.08 m/sec).

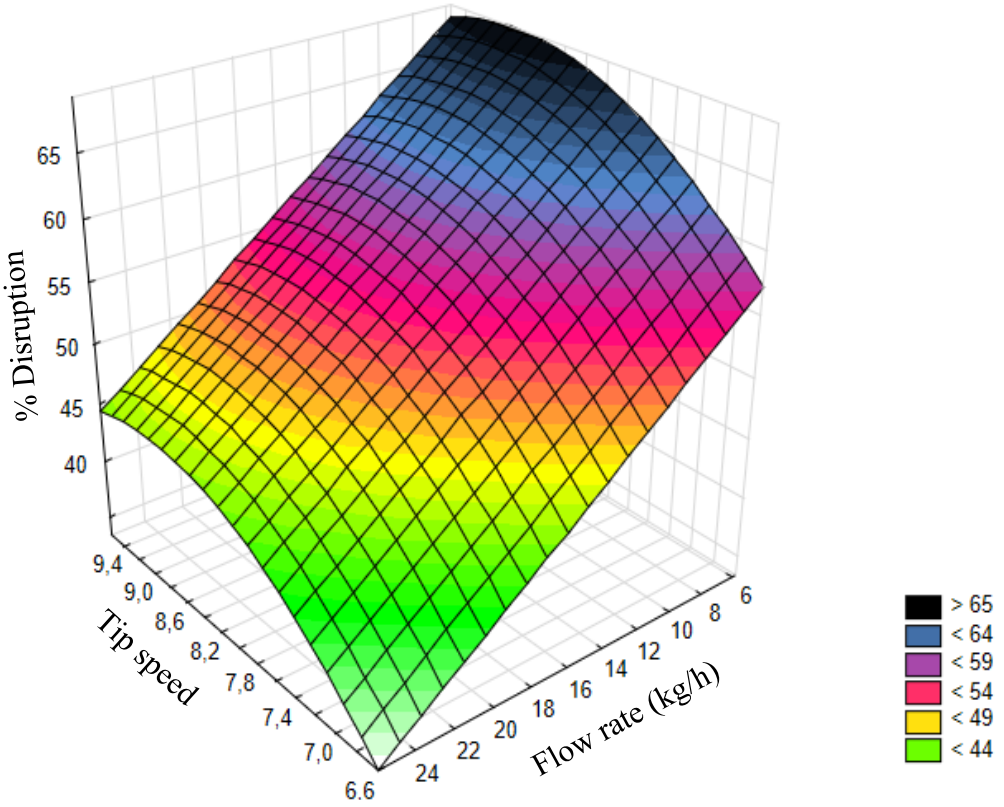

Figure 23: Disruption efficiency of *P. tricornutum* by bead milling with Zr beads. Effect of flow rate (kg/h) and tip speed (m/sec). The third variable (Dry matter) is set as mean experimental value (16.64%).

In general, many studies have concluded that bead milling is an efficient cell wall disruption method (Doucha and Livansky, 2008; Günerken et al, 2015; Safi et al., 2017) but there are also studies, like that of Zheng et al. (2011) in *Chlorella. vulgaris*, which showed limited nutrient release after bead milling. The reason may lay in the fact that in the latter study applied the authors tested different and limited processing parameter settings. This demonstrates the need for optimization of the cell wall disruption processing for each species and different method applied.

In our bead milling study with small glass beads, *P. tricornutum* biomass disruption increased with raised pump flow rate and medium-high tip speed; but taking into account the model plot (Figure 21) of surface response analysis, potentially the maximum disruption could be achieved using a lower tip speed. Also, in the second study with the small Zr beads, the combination of higher DM content with lower tip speed (~9 m/sec), could give the optimal disruption for *P. tricornutum* biomass.

Apart from % disruption efficiency, in this second study, regression analysis with backward removal was performed for % aerobic bacteria reduction (Appendix 10), viscosity alteration (Appendix 11), % fat (Appendix 12) and % soluble protein (Appendix 13) release. % Soluble protein release was not correlated with bead milling (R^2=0) and the reason may lie in the erratic measurements of the central and star points (Appendix 1) as a result of the inadequacy of the BCA method to determining the total concentration of protein in our samples. % Fat release in significantly (R^2=0.756) correlated with bead milling parameters, and specifically positively correlated with FR, and slightly negatively by the interaction of DMxFR. Fat is released better at higher flow rates, but only when combined with lower biomass dry matters (Figure 24).

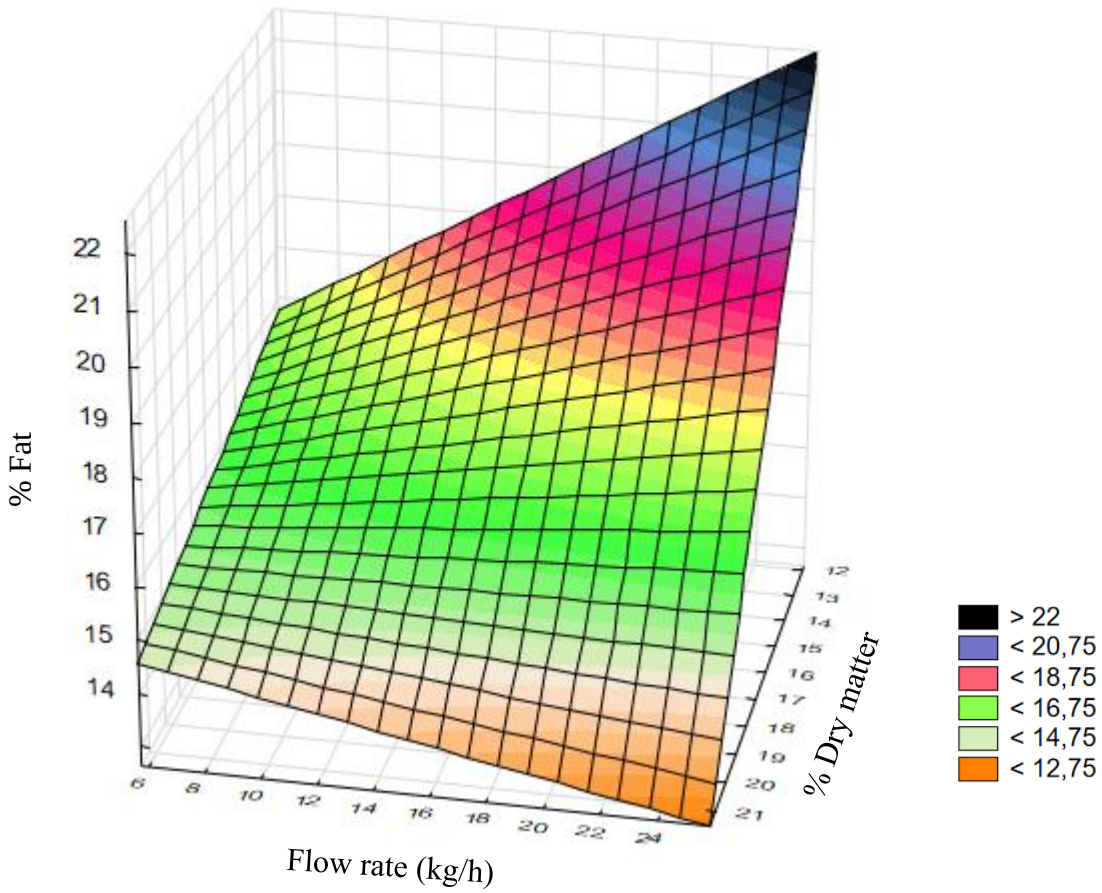

Figure 24 : % Fat release from *P. tricornutum* by bead milling with Zr beads. Effect of flow rate (kg/h) and dry matter (%)

Aerobic bacteria reduction was significantly affected by FR and the interaction of DMxFR (Table 11). Higher reduction was achieved on, high flow rate but relatively low biomass dry matter (Figure 25); which comes in contrast to our previous correlation of high DM with more efficient disruption. Reduction of accompanying microorganisms in the microalgae biomass by bead milling, was observed also, by Kokkali et al. (2018b) on a disintegration study of *Nannochloropsis sp.* as well as, by Doucha and Livansky (2008) on study with *Chlorella sp.*.

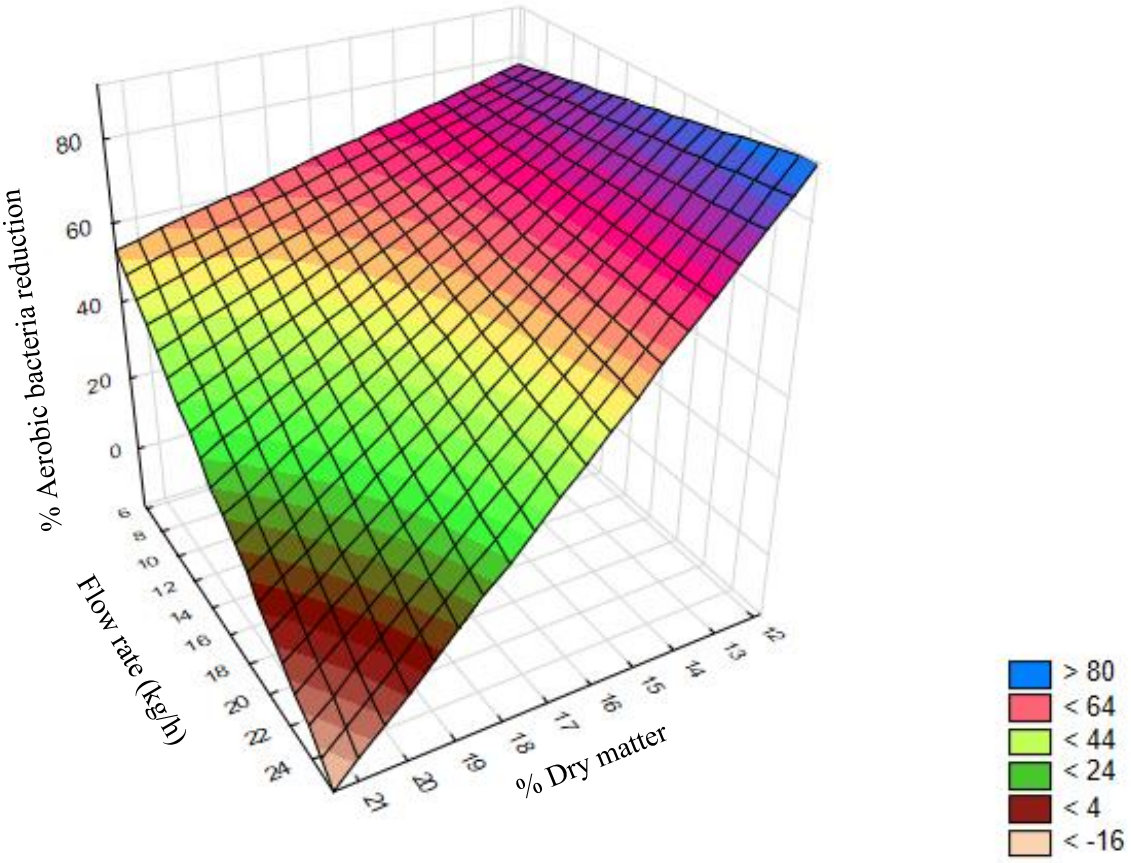

Figure 25: (%) Aerobic bacteria reduction on *P. tricornutum*. by bead milling with Zr beads. Effect of flow rate (kg/h) and dry matter (%).

The study of Doucha and Livansky, supported that bead milling with the use of glass beads, increased the microorganism reduction. This finding comes in contrast to Molina Grima et al. (2004) study, in which microorganism disintegration was doubled after using Zr beads. The conflicting results of the two aforementioned studies were ascribed to the density of Zr beads and their expediency on viscous substances. Microbial suspensions have lower viscocity thus glass beads yield better results (Schütte and Kula, (1990) as cited in Doucha and Livansky 2008). Effects of mass viscosity during bead milling may also provide explanation to our finding that higher aerobic reduction is achieved at lower biomass dry matter. When measured, we saw that biomass viscosity increased linearly with increasing biomass dry matter (Figure 26). Moreover, samples

with increased % disruption following bead milling had significantly higher viscosity (0.7 to 5 times higher) as compared to unprocessed biomass (*P. tricornutum*) biomass of the same dry matter but in disrupted biomass viscosity did not follow the dry matter levels in a canonical way. Using response surface analysis, we found that viscosity was significantly affected (R^2= 0.874) by bead milling parameters; namely, flow rate and the interaction of tip speed with both flow rate and dry matter (Figure 27). High flow rate and tip speed combined with biomass of lower dry matter predicts in the highest resulting viscosity level of disrupted *P. tricornutum* biomass.

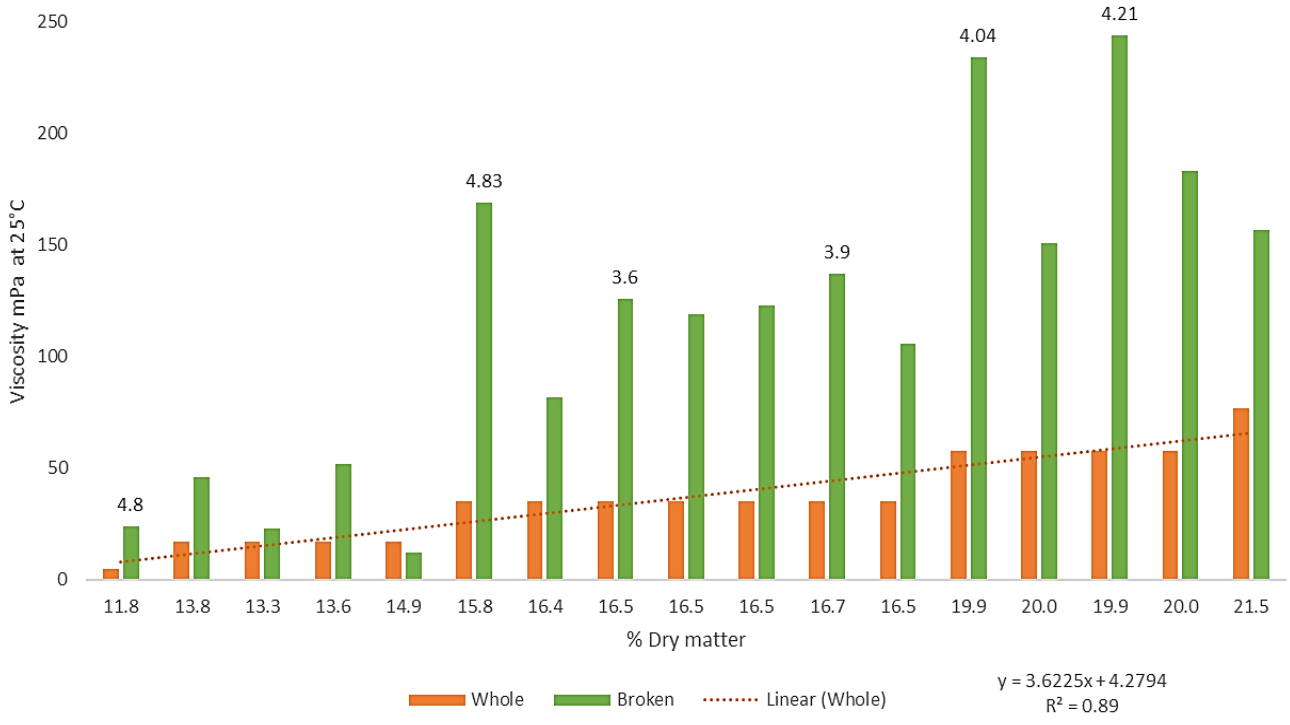

Figure 26: Viscosity alterations on whole and broken *P. tricornutum* biomass of different DM levels.

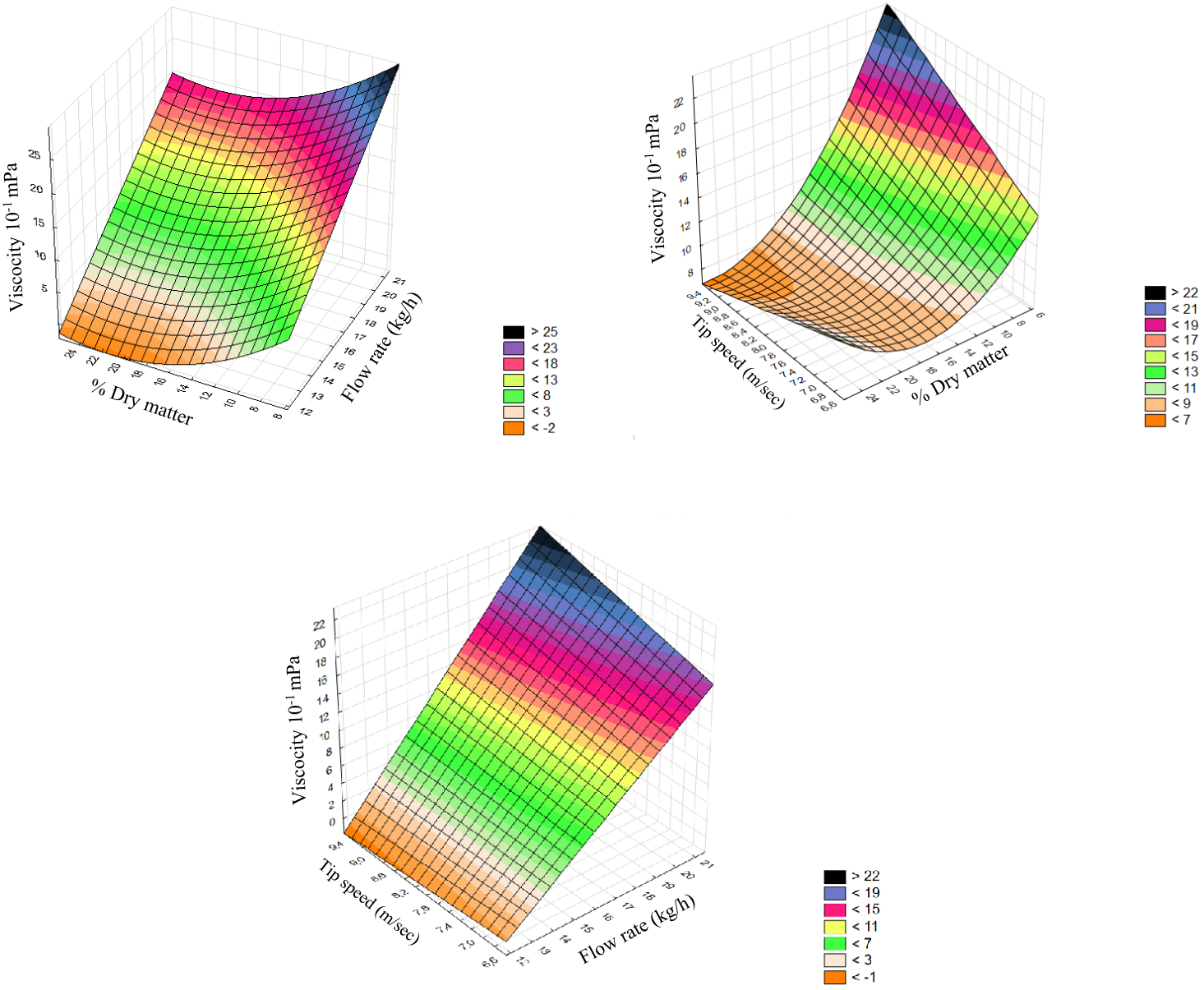

Figure 27: Viscosity (×10^{-1} mPa) alteration by bead milling of *P. tricornutum* biomass with Zr beads. The third variable for each graph (i.e Dry matter, Flow rate, and Tip speed) is set as mean experimental value (16.64%, 15.76 kg/h and 8.08 m/sec, respectivelly).

As seen before, higher flow rate leads to less efficient cell wall disruption. In parallel, higher viscosity levels are observed on broken biomass of lower dry matter at higher flow rates. Finally, highest aerobic bacteria reduction was achieved in biomass of lower dry matter at higher flow rates. Thus, it appears that bacteria reduction may positively correlate with mass viscosity increase. Reiterating the interpretation of Doucha and Livansky (2008), high density beads (i.e. Zr *vs* glass) have advantage on high viscous suspensions.

Regarding microalgae cell disruption efficiency, we did not achieve maximum disruption of *P. tricornutum* biomass in neither of our studies. *P. tricornutum* has different cell morphotypes (i.e. fusiform, triradiate, and oval), as do also bacterial *vs* microalgae cells, which showed different behavior and cell disruption results under bead milling. It may thus be the case that the tested disruption processes and constructed models were not optimal for each one and all three morphotypes combined, resulting in limited disruption efficiency in this species' biomass. The Zr beads we used had 0.3mm diameter, while glass beads had 0.25-0.4mm diameter. On average the area of each beads is approximately 0.3 mm^2, whereas *P. tricornutum* cells have an average surface area of 45-100 μm^2 or approximately 3 to 7 times smaller than the beads. Thus, the space created between adjacent beads may allow some cells to "escape" and remain intact. SEM pictures in figure 28 illustrate intact and disrupted cells of *P. tricornutum* after bead milling with small glass beads. In Figure 28b and 28d we probably see the triradiate morphotype of *P. tricornutum* intact and disrupted, respectively; while figure 28e illustrates embedded cell fractions.

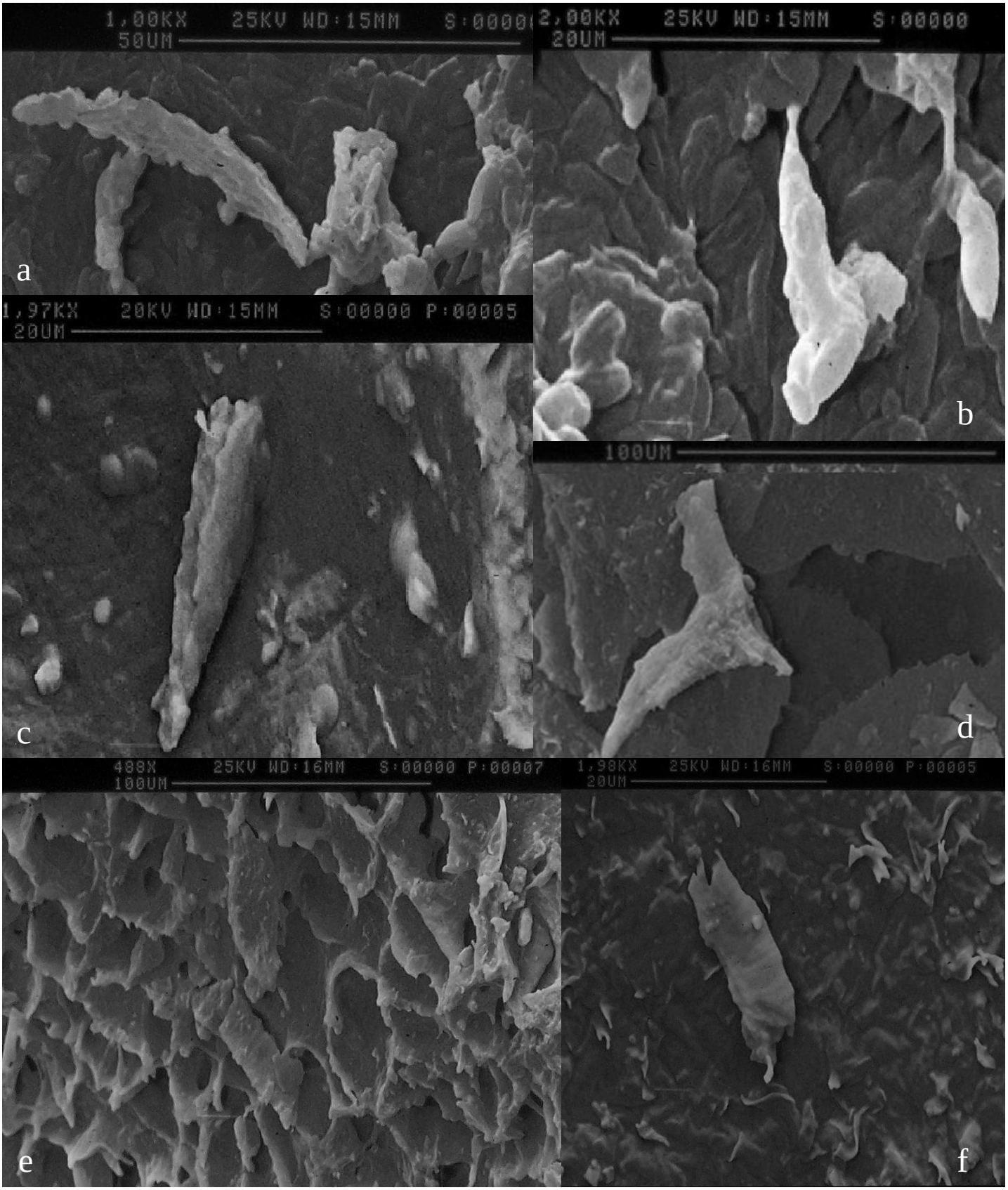

Figure 28: *P. tricornutum* cell observed under SEM. a) and b) intact cells. c) d) e) and f) disrupted by bead milling with small glass beads.
Scale bar on top of each picture set.

3.1.2a Interrelation of nutrient extraction with disruption efficiency

In our study, we saw higher lipid extraction efficiency after bead milling compared to non-processed biomass but no alteration on sum of ω-3 PUFA, as well as EPA and DHA extraction (Figure 29). Values for whole *P. tricornutum* biomass are from different batches of delivered biomass; whereas for disrupted are from our *P. tricornutum* bead milling processing trials.

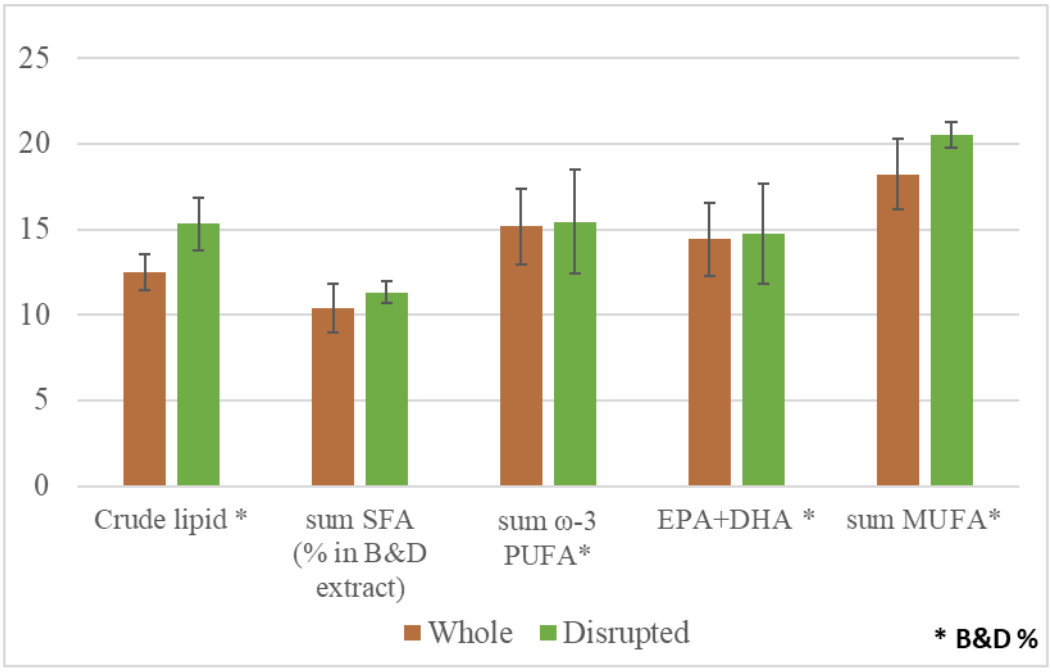

Figure 29: Crude lipid, sum of: SFA, ω-3, and MUFA, and EPA+DHA content of whole and cell wall disrupted *P. tricornutum* biomass.

In the bead milling study with small glass beads, higher soluble protein release was achieved on disrupted specimens; the higher value, 55.5 % soluble protein of total protein content, was detected on the sample with 65% cell wall disruption degree (Figure 30). The same sample had the highest analyzed total antioxidant capacity (TAC), of the above specimens; whereas the differences of TAC among them were minor. As also

observed in our studies with *T. chuii*, disrupted *P. tricornutum* samples had increased TAC in relation to the intact sample (Figure 31).

Antioxidant capacity of *P. tricornutum* biomass was also studied by Ahmed et al. (2014), with the oxygen radical absorbance capacity (ORAC) method. Approximately 350 μmol trolox Equivalent /g DW was obtained after water extraction of the studied biomass. In their study, Goiris et al. (2012) used the TEAC method to determine total antioxidant capacity (TAC) on differently cultured batches of *P. tricornutum* biomass and measured average values of 19.1±0.45 μmol trolox/g DW. In our study (with the use of TEAC method) we measured TAC values of 27 ± 0.44 mM trolox/g DW in disrupted and 22.4 ± 0.12 mM trolox/g DW in non-disrupted biomass. Comparing the results of the three studies we see, that Ahmed et al. (2014), showed significantly higher antioxidant capacity of *P. tricornutum* biomass than ours and Goiris et al. (2012) results. The reason may lie on the sensitivity of the two methods as long as the cultivation techniques of the biomass. In Goiris et al. (2012), total phenolic content was found to be on average 3.5±0.45 mg Gallic acid g^{-1} DW, whereas in our study, total phenolic content was found more than 30 times higher. Disrupted samples showed increased phenolic content compared to, non-disrupted ones, and in average 148±4 mg Gallic acid/g DW (Figure 32). Both TAC and TPC of *P. tricornutum* biomass, measured in our and the aforementioned studies showed great variation; more experiments need to be performed for understanding which methods to be used and for obtaining systematic results.

Carotenoid content of *P. tricornutum* biomass after bead milling with small glass beads fluctuated (Figure 32). Namely, disrupted biomass in general had increased carotenoid concentration, but the 48% and 73% disrupted samples, lacked chlorophyll-b

and total carotenoid content, respectively. Denaturation of the pigments may have occurred during biomass disintegration or drying. Thermal denaturation, as well as denaturation due to mechanical forces is a common risk in microalgae processing (Doucha and Livansky 2008; Pasquet et *al.*, 2010).

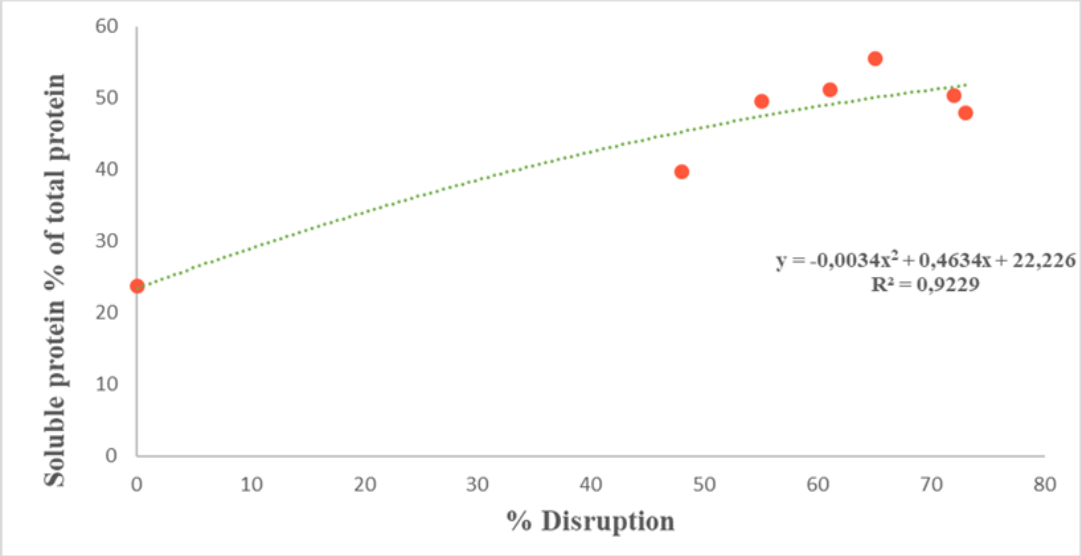

Figure 30: Correlation of cell wall disruption of *P. tricornutum* by bead milling with glass beads, and the released soluble protein.

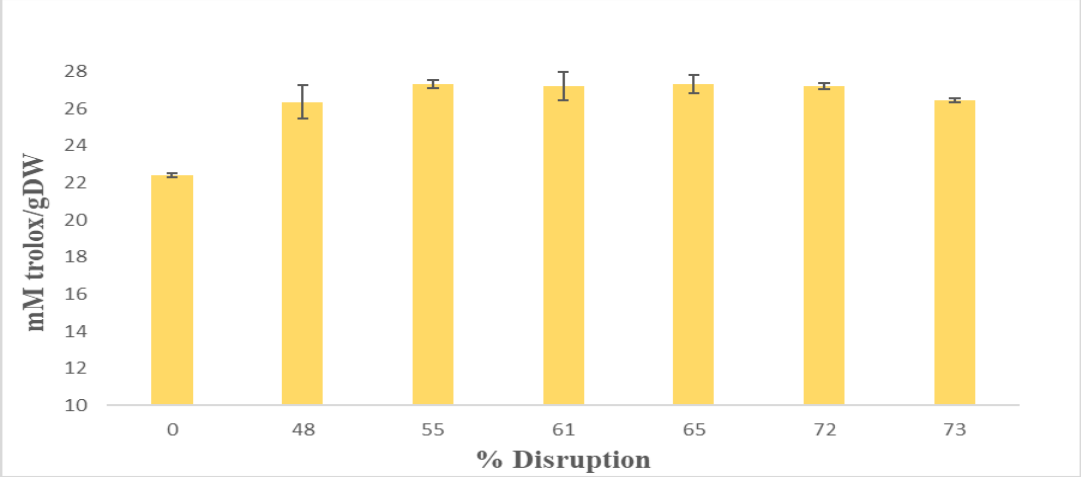

Figure 31 Total antioxidant capacity of *P. tricornutum* biomass, after bead milling with small glass beads.

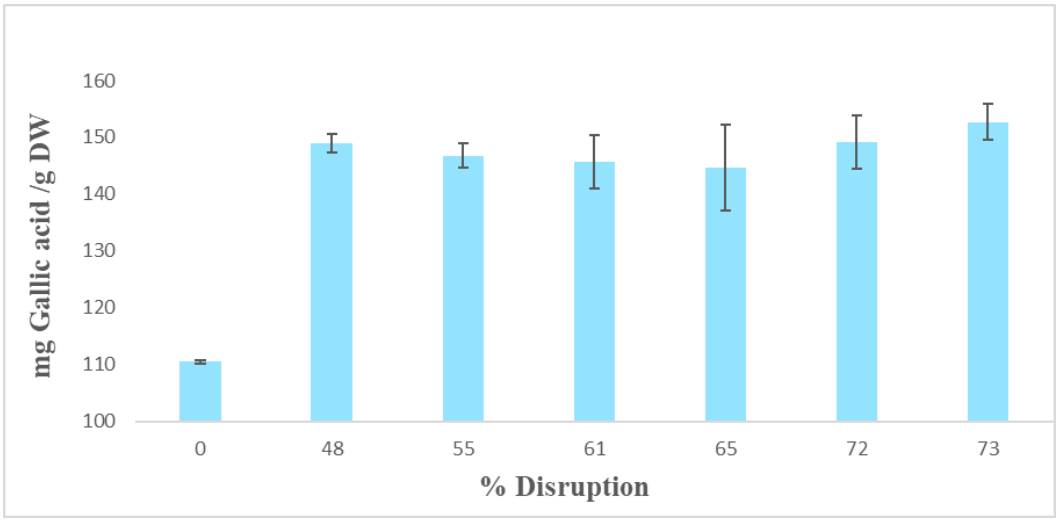

Figure 32: Total phenolic compounds of *P. tricornutum* biomass, after bead milling with small glass beads.

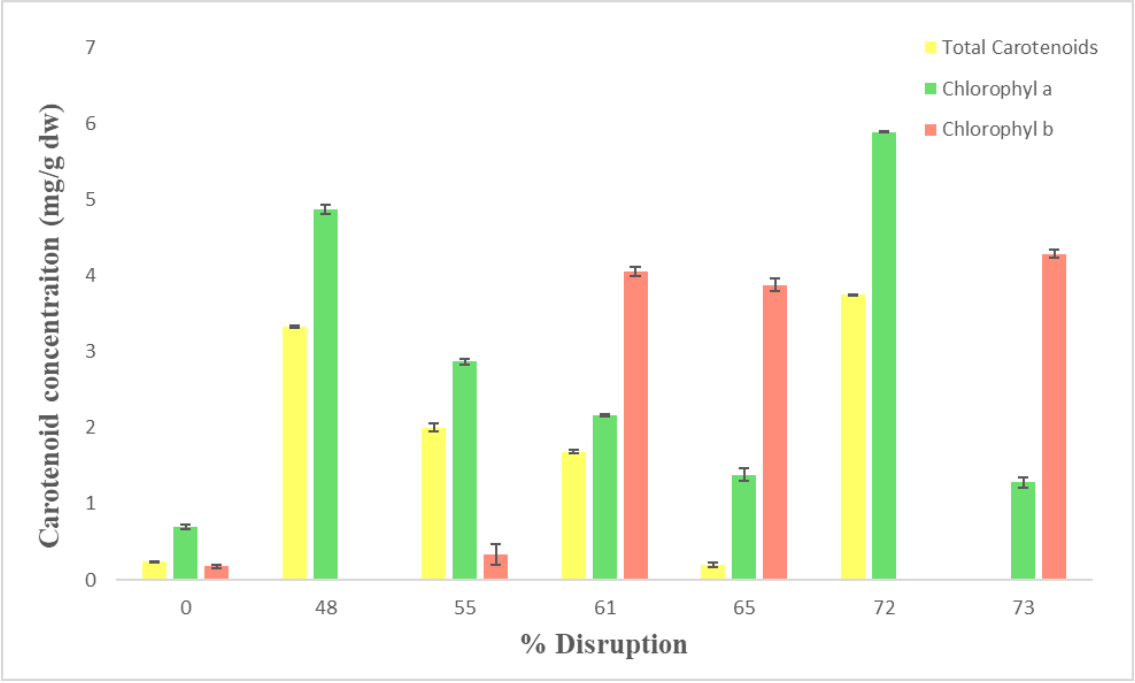

Figure 33: Carotenoid content of *P. tricornutum* biomass, after bead milling with small glass beads.

3.2 PEF treatment on T. chuii and P. tricornutum biomass for enhancement of nutrient extraction

In our experiment, PEF specific energy input varied from 50 to 300 kJ/kg; the number of pulses from 23 to 1200, depending on the voltage applied, while field strength was fixed. From the 24 (2x12) different runs per species that were carried out (see Table 3). Based on literature evaluation and previous experience (personal communication with Prof. F. Barba), Run 2 and 10 were chosen for further investigation of nutrient extraction, as these conditions had relatively low specific energy (~100 KJ.kg), and specific energy input influences the degree of membrane permeabilization (Frey, Gusbeth & Schwartz 2013; Goette *et al.*, 2013).

In our study, we investigated the effects of PEF as pretreatment, as well as the effects of extraction time and solvent type (henceforth 'treatment') on nutrient extraction from two microalgae species (i.e. *T. chuii* and *P. tricornutum*). Multivariate General Linear Model (GLM) analysis revealed that the extraction of most of the nutrients studied was significantly affected ($P \leq 0.05$) by the applied treatments on the microalgae biomasses. Extraction of total carotenoids and chlorophyll a, as well as total antioxidant capacity (ABTS radical), were significantly affected by all varied treatment parameters (species, time, solvent, PEF) and their interactions. Extraction of chlorophyll b was significantly affected by extraction time and solvent type, and several parameter interactions. Last, extraction of total phenolic compounds was significantly affected by species, solvent and PEF and several parameter interactions (Table 12).

Table 12: Effects of PEF, solvent treatment and incubation time on TAC and analyzed levels of bioactive compounds in freeze dried *T. chuii* and *P. tricornutum* biomass (ChlorA: Chlorophyll A in in mg/g, ChlorB: Chlorophyll B in in mg/g, Carotenoids: Total carotenoids in mg/g, TAC: Total antioxidant capacity in mM trolox/g, Phenolics: Total phenolic compounds in mg GAE/L) (values are means and standard error of the means – SEM; n=2 x 3 analytical replicates)

Tetraselmis chuii																				
PEF	No pre-treatment				**PEF1** 30 kV 45 pulses				**PEF2** 10 kV 400 pulses				SEM	Treatment	PEF	Solvent	Time	PEF* Solvent	PEF* time	Solvent *time	PEF* Solvent *time
Time (h)	4h		24h		4h		24h		4h		24h			P value							
Solvent	water	dmso	water	dmso	water	dmso	water	dmso	water	dmso	water	dmso									
ChlorA	0.155^{b}	0.53^{f}	0.195^{c}	0.625^{g}	0.18^{c}	0.69^{h}	0.28^{d}	0.695^{hi}	0.02^{a}	0.71^{ij}	0.34^{e}	0.715^{j}	0.075	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ChlorB	0.55^{bc}	0.33^{ab}	0.2^{ab}	0.39^{abc}	0.18^{ab}	0.43^{abc}	0.79^{c}	0.47^{bc}	0.58^{bc}	0.18^{ab}	0.43^{abc}	0.00^{a}	0.062	0.05	0.10	0.03	ns	ns	0.07	ns	ns
Carotenoids	0.35^{b}	0.15^{d}	0.35^{b}	0.18^{e}	0.30^{b}	0.175^{e}	0.00^{a}	0.175^{e}	0.00^{a}	0.395^{f}	0.10^{c}	0.48^{g}	0.044	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
TAC	37.15^{f}	34.47^{d}	29.04^{b}	48.1^{i}	40.14^{g}	44.85^{h}	21.13^{a}	49.27^{j}	44.625^{h}	51.97^{k}	31.565^{c}	34.97^{e}	2.666	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenolics	198.55^{a}	243.21^{f}	241.8^{f}	248.16^{g}	261.58^{h}	270.1^{j}	222.79^{c}	264.52^{i}	200.16^{a}	216.91^{b}	229.25^{d}	237.48^{e}	6.819	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Phaeodactylum tricornutum																				
PEF	No treatment				**PEF1** 30 kV 45 pulses				**PEF2** 10 kV 400 pulses				SEM	Treatment	PEF	Solvent	Time	PEF*Solvent	PEF* time	Solvent *time	PEF* Solvent *time
Time (h)	4h		24h		4h		24h		4h		24h			P value							
Solvent	water	dmso	water	dmso	water	dmso	water	dmso	water	dmso	water	dmso									
ChlorA	0.00^{a}	2.64^{e}	0.14^{bc}	3.715^{g}	0.11^{b}	4.42^{g}	0.23^{d}	2.64^{e}	0.16^{c}	3.95^{h}	0.155^{c}	3.42^{f}	0.52	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ChlorB	0.65^{f}	0.93^{h}	0.30^{c}	0.00^{a}	0.17^{b}	0.00^{a}	0.36^{d}	0.00^{a}	0.51^{e}	0.87^{g}	0.51^{e}	0.00^{a}	0.10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Carotenoids	0.00^{a}	0.96^{c}	0.00^{a}	1.315^{f}	0.3^{b}	1.5^{h}	0.00^{a}	1.40^{g}	0.00^{a}	1.12^{d}	0.00^{a}	1.28^{e}	0.19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TAC	18.97^{c}	52.61^{k}	17.34^{b}	52.27^{j}	32.24^{g}	40.92^{h}	15.58^{a}	51.36^{j}	19.41^{d}	28.90^{e}	15.78^{a}	29.88^{f}	4.24	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Phenolics	125.42^{a}	286.7^{c}	160.77^{ab}	314.74^{c}	180.76^{b}	307.72^{c}	158.43^{ab}	327.41^{c}	196.93^{b}	307.42^{c}	149.91^{ab}	301.84^{c}	24.78	0.000	ns	0.000	ns	0.000	ns	ns	ns

* Values not sharing common superscript letters are significantly different by ANOVA and Duncan post hoc test ($P<0.05$). ** ns, non significant.

In *T. chuii*, significantly more chlorophyll a was extracted by PEF2, with the use of DMSO solvent (1.184±0.004 mg chlorophyll a /g DW), while incubation time did not have a significant effect (4h: 0.71±0.07 mg chlorophyll a /g DW *vs* 24h: 0.715±0.07 mg chlorophyll a /g DW). 4.42±0.52 mg of chlorophyll a /g DW of *P. tricornutum,* was extracted under PEF1, with the use of DMSO solvent after 4h incubation.

In *P. tricornutum,* PEF did not affect the analyzed levels of chlorophyll b significantly. The highest analyzed level of chlorophyll b was 0.93±0.1 mg /g DW when no PEF treatment was applied. In *T. chuii*, the highest amount of chlorophyll b (0.79±0.06 mg /g DW) was analyzed following PEF1, using of water solvent during 24 h incubation.

Maximum amount of total carotenoids for both species were analyzed in samples incubated for 24h, using DMSO as solvent. PEF1 was the most efficient PEF treatment for *P. tricornutum* (maximum concentration obtained: 1.395±0.19 mg of Carotenoids /g DW), whereas PEF2 was the most efficient PEF treatment for *T. chuii* (maximum concentration obtained: 0.48±0.04 mg of Carotenoids /g DW).

Total antioxidant capacity in *P. tricornutum* was not affected by PEF, as a maximum concentration of 52.61±4.24 mM trolox/gDW, was analyzed in non-PEF treated samples. For *T. chuii*, 51.97±2.67 mM trolox/gDW was the maximum extracted TAC measured, obtained after 4h incubation, using DMSO as solvent and PEF2 as pretreatment.

Higher levels of total phenolic compounds in both species were extracted after 4h incubation, using DMSO solvent. PEF1 was the most efficient PEF treatment for *T. chuii* (maximum levels analyzed: 270.1± 6.82 mg Gallic acid /g DW), whereas in the case of

P. tricornutum it was PEF2 that was the most efficient PEF pre-treatment (maximum levels analyzed: 307.42±24.78 mg Gallic acid /g of DW).

General Linear Model (GLM) multivariate analysis for *T. chuii* revealed significant effects ($P \leq 0.05$) of the treatment parameters on almost all target substances analyzed. Extraction time, PEF, as well as their interactions affected significantly nutrient extraction of all the dependent variables apart from the analyzed levels of chlorophyll b; whereas solvent, and its interactions with the other treatment factors affected significantly all the dependent variables. PEF0 and PEF1 treatment, showed no significant difference on carotenoid extraction, but the extraction of all the other substances were affected significantly by PEF pretreatment. Moreover, based on GLM multivariate analysis we also saw significant effects ($P \leq 0.05$) of the different treatment parameters on all extracted substances from the *P. tricornutum* samples, apart from TPC. Specifically, incubation time, solvent type, PEF, as well as their interactions affected significantly nutrient extraction of all the dependent variables (TPC excluded). Extraction of TPC after PEF treatment was significantly affected by solvent type, treatment, as well as the interaction of PEF treatment and solvent.

Taking into account all the above, we cannot conclude which treatment is generally the best, as different nutrients require different treatments for different species. Grimi et al. (2014), after using PEF treatment on *Nannochloropsis sp.* found no significant difference on pigment extraction. On the contrary, Parniakov et al. (2015), found incresed pigpment yield after pretreatment of *Nannochloropsis sp*. with PEF and binary mixture of organic solvents. Even though both studies investigated the correlation of PEF and pigment extraction on the same microalgae species their results were

conflicting. Both studies used the same PEF field strength and specific energy (20 kV/cm, 13.3–53.1 kJ/kg), voltage and number of pulses were not mentioned. The conflicting results of the two studies may lie on the fact that the latter after PEF treatment used the binary organic mixture of solvent for enhancement of extraction. In another study, Töpfl (2006) found increased yield of pigments (chlorophyll and carotenoid content) in *Spirulina and Chlorella sp.* after applying PEF treatment without use of extraction solvents. In his study Töpfl (2006) applied different field strength and specific energy from the other two aforementioned studies (15 kV/cm, 100 kJ/kg); while the studied microalgae species differed.

The effects of extraction time on the analyzed nutrient levels can be seen in Figure 34. The highest analyzed values of chlorophyll a, chlorophyll b and TAC in crude extracts of both studied species were at 4 hours incubation time. Contrarily, total carotenoid levels were higher following 24, as compared to 4 hours incubation time. In our study, the extraction of TPC was not significantly affected by incubation time. In the study of Chew et al. (2011), incubation time had also signidficantly affected the measured TAC in *Centella asiatica* extracts reaching highest levels after 2 hours of incubation.

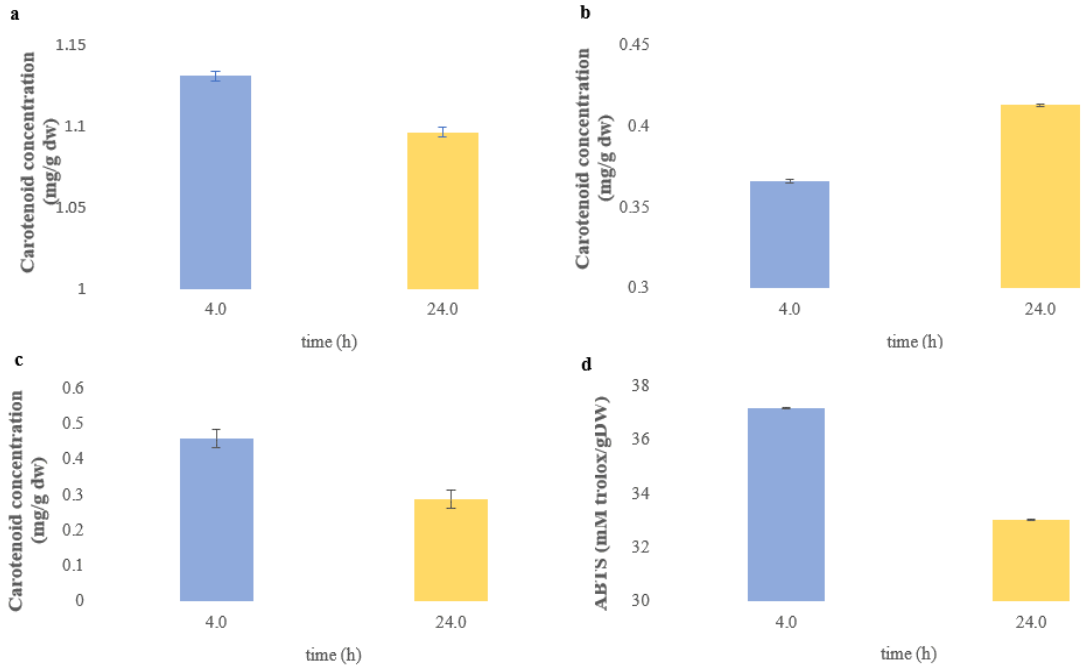

Figure 34: Effect of incubation time on analyzed a) Chlorophyll a, b) total carotenoids and c) Chlorophyll b (in mg carotenoids /g DW biomass), and d) TAC by the ABTS assay (in mM trolox/gDW)).
Values are means ± standard deviation

Solvent type significantly affected the extraction of all studied compounds (Figure 35). DMSO was in all cases exept from chlorophyll b the most efficient solvent. Parniakov et al. (2015) concluded that the most effective extraction of target components from *Nannochloropsis sp.* was achieved with a DMSO binary mixture of organic solvents. While in their review, Barba et al. (2014) notes that PEF combined with solvent extraction using DMSO, is a useful tool for the recovery of valuable compounds from different matrices.

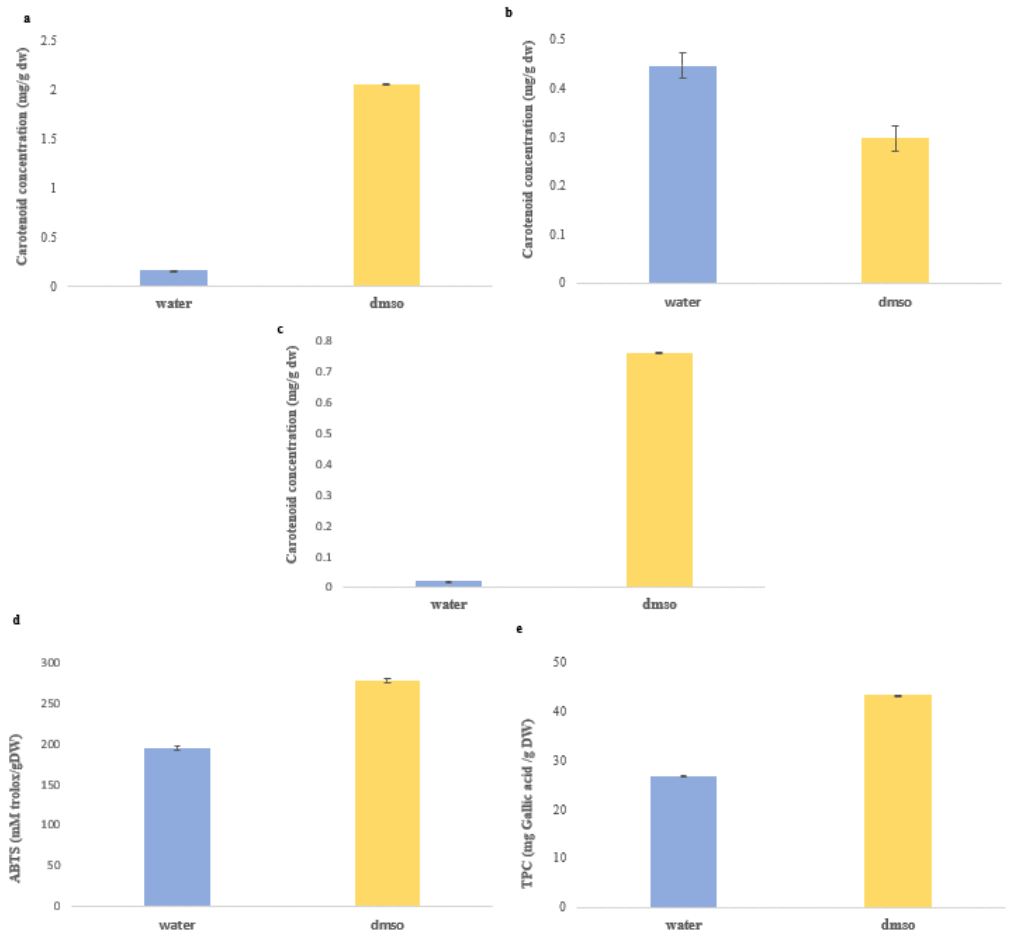

Figure 35: Effect of solvent type on analyzed a) chlorophyll a, b) chlorophyll b, and c) total carotenoids (in mg carotenoids /g DW biomass), and d) TAC (ABTS assay in mM trolox/gDW), e) Total phenolic compounds (TPC in mg Gallic acid /g DW). *Values are means ± standard deviation*

Pre-treatment of microalgae biomass with PEF in two different voltage/pulses combination significantly affected the extraction of all studied components except from chlorophll b. PEF pretreatment enhanced the extraction of the target components compared to non treatment application, regardless the conditions used (voltage/pulses) (Figure 36). TAC and TPC concentration were significantly higher in samples treated at higher voltage and less pulses (PEF1), while combination of lower voltage with greater number of pulses (PEF 2) enhanced carotenoid concentration (both chlorophyll a and

total carotenoids). For carotenoid extraction along with the plasma membranes, the chloroplast membranes has to be electroporated as carotenoids are bound to chloroplasts (Poojary *et al.*, 2016). Thus, higher external electric field strengths are required for permeabilization of smaller internal organelles like chloroplasts (Esser *et al.*, 2010). Our findings are in contrast with all the above as field strength at PEF1 was high=er than PEF2 (3kV/cm and 1 kV/cm, respectively) but higher carotenoid extraction was observed by PEF2 treatment.

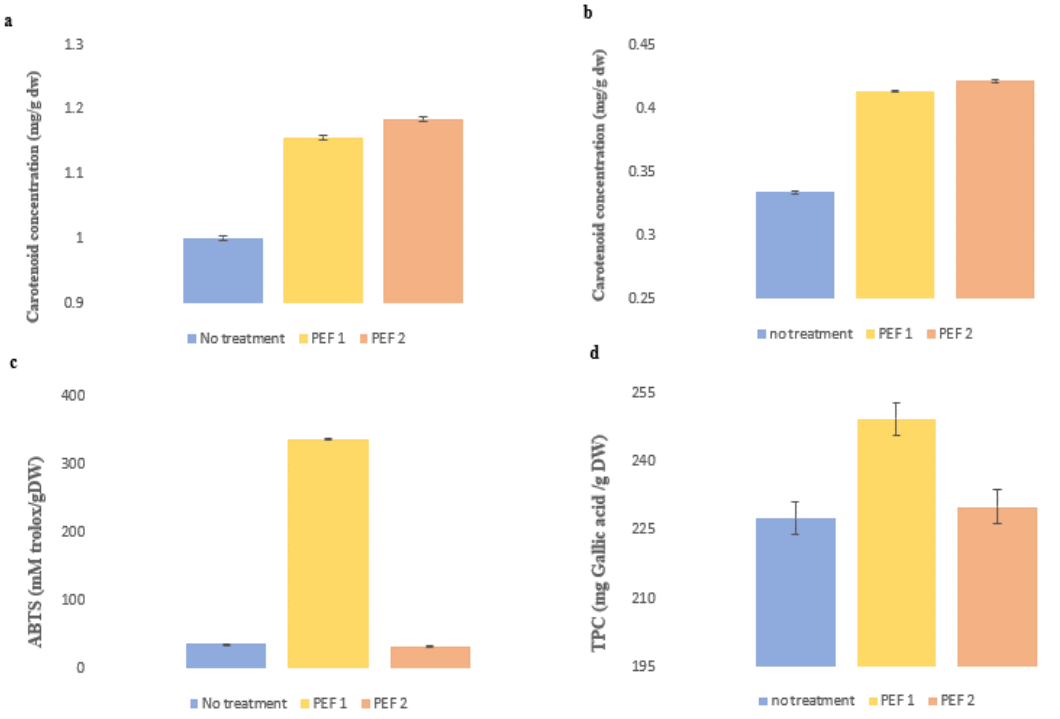

Figure 36: Effect of PEF on analyzed a) chlorophyll a and b) total carotenoids (in mg carotenoids /g DW biomass), c) TAC (by ABTS assay in mM trolox/gDW) and d) TPC (mg Gallic acid /g DW).
Values are means ± standard deviation; PEF1: Voltage 30kV and 45 pulses; PEF2: Voltage 10kV 400 pulses

Chemical comparison of the two studied species (Figure 37) showed that *P. tricornutum* biomass had significantly higher carotenoid concentration; 1.8± 0.003 mg chlorophyll a /g dw and 0.6±0.001 mg of total carotenoids /g dw; while, *T. chuii* had significantly higher TAC (ABTS^{+}) 38.9±0.026 mM trolox/g dw. These values are means of the total extracted substances after different PEF treatment and extraction techniques; but the differences are attributed to the initial different composition of the two species.

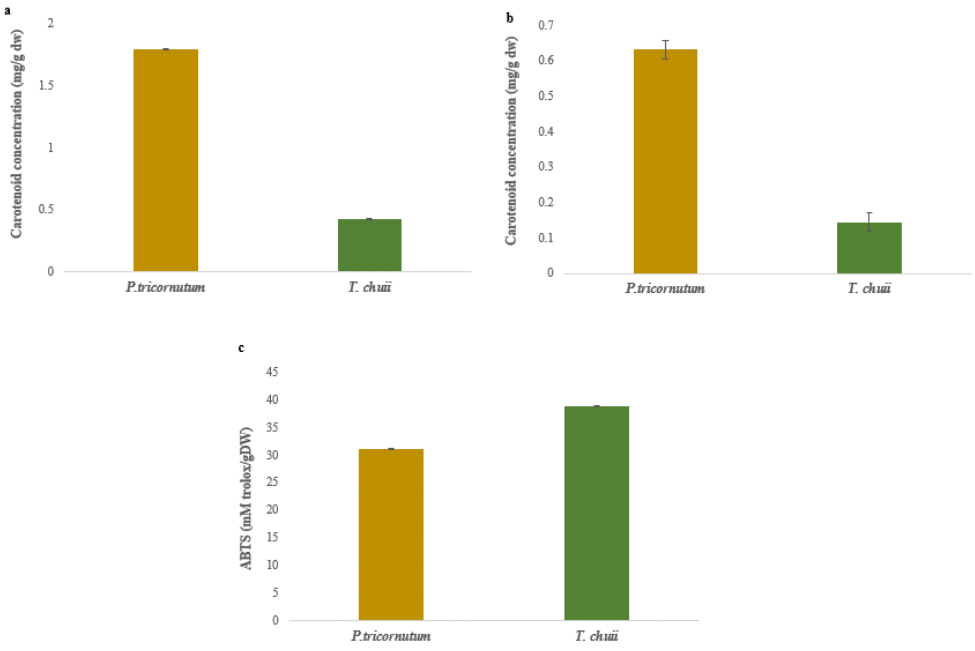

Figure 37: Analyzed levels of a) chlorophyll a, and b) total carotenoids (in mg carotenoids /g DW biomass), c) TAC (ABTS assay in mM trolox/gDW), in processed *P. tricornutum* and *T. chuii* samples.
Values are means ± standard deviation

4.0 Conclusion

In the present study we worked towards optimization of bead milling parameters for efficient disruption of *Tetraselmis chuii* and *Phaeodactylum tricornutum* cell walls. Maximum disruption efficiency (99%) of *T. chuii* was achieved by bead milling the biomass with small glass beads, with combination of high dry matter content (22.5%), high agitator tip speed (12 m/sec), and low flow rate (3.6 kg/h). Analysis of nutrients and bioactive compounds, on disrupted and non-disrupted samples of *T. chuii*, showed that disruption of cell wall of *T. chuii* increases the extracted levels of the nutrients and bioactive substances. Using bead milling with small glass beads, we reached 73% disruption of *P. tricornutum* biomass, without affecting total levels of EPA and DHA. Using Zr beads, the maximum disruption achieved was 86%. Besides (%) disruption, (%) fat release, (%) aerobic bacteria reduction, and viscosity were also significantly affected by the used bead milling parameters.

Pulse electric fields was also tested as an alternative pre-treatment method for the enhancement of bioactive compounds extraction from *T. chuii* and *P. tricornutum* biomasses. PEF treatment, solvent type and extraction time affected significantly the extraction of carotenoids and phenolic compounds; as well as the total antioxidant capacity of the biomass.

Our studies demonstrate that bead milling is an efficient disruption method for *T. chuii* and *P. tricornutum* biomass, and that PEF may be a promising alternative for enhancement of bioactive compound extraction.

5.0 Recommendations associated with this study

First and foremost, I would like to emphasize that in the present study, when microalgae biomass was pipped for analysis, positive displacement pipettes were used. In the beginning of the experiments, a normal air pipet was used, but we saw that due to the viscous nature of the microalgae samples, the set volume was not sucked in accurately. I had to reassess more than 100 samples, with the new pipettes. Also, while bead milling, we came across difficulties with the set and actual flow rates. A more powerful pump maybe would give more systematic results on flow rate.

From the obtained results, we saw that disruption efficiency by bead milling is different between species; thus, further optimization studies on different species should be performed. Also, for *Phaeodactylum tricornutum*, further study on cry matter and tip speed extreme values, could shed light on the complex outcomes of this study, and maybe would maximize the disruption efficiency of it. For time and biomass saving reasons not all bead milling parameters, were tested in the current study. Different bead size, use of accelerator instead of a conventional agitator, maximum bead filling of the chamber, are some bead milling parameters which could be further studied towards a more complete optimization study. Combination of Pulse Electric Fields and bead milling may also result on better nutrient and bioactive compounds, extraction.

Last, I would like to highlight the necessity to follow systematic methodology when studying processing parameters and interpreting the disruption efficiency using bead milling. During literature evaluation, we came across repeated (and apparently suboptimal) procedures across different studies compromising the respective conclusions.

6.0 Publications associated with this thesis

Oral presentations in conferences

Nunes, M.C., Vasco, I., **Kokkali, M.,** Kousoulaki, K., Sousa, I., Raymundo, A., 2019. Improving gluten free bread bioactivity with *Tetraselmis chuii* disrupted biomass. ALGAEUROPE 2019, 03-05 December 2019, Paris, France.

Kousoulaki, K., **Kokkali, M.**, Sørensen, M., Kleinegris, D., Kiron, V., Thoresen, L., 2019. Optimalisering i nedstrømsprosessering av mikroalger dyrket fototrofisk for at de skal best bidra med mer enn protein og marine fettsyrer i fiskeoppdrett. Det 14. Norske Fiskeernæringsseminaret, 13-14 November 2019, Solstrand, Norway.

Kokkali, M., Barba, F.J., Marti-Quijal, F.J., Kleinegris, D., Kousoulaki, K., 2019. Release of phenolics and other antioxidants from microalgae *Phaeodactylum tricornutum* and *Tetraselmis chuii* following bead milling. Oral presentation in Aquaculture Europe 2019, 7-10 October 2019, Berlin, Germany.

Sørensen, M., Kousoulaki, K., Kleinegris, D., Johnsen, C.A., Palihawadana, A.M., **Kokkali**, **M.**, Kiron, V., 2019. Whole or broken cells of *Phaeodactulum tricornutum* and *Tetraselmis chuii* fed to Atlantic salmon *Salmo salar*. Oral presentation in Aquaculture Europe 2019, 7-10 October 2019, Berlin, Germany.

M.E. Kokkali, K. Kousoulaki, 2019. Biorefinery of high nutritional value microalgae, *Tetraselmis chuii* and *Phaeodactylum tricornutum*; for sustainable aquafeed production. Oral presentation at the VI International Student Congress of Food Science and Technology held in Valencia, 21st-22nd February 2019. ***Best presentation award.***

Kokkali M.E., Kleinegris, D.M.M., de Vree J.H., Haugsgjerd B.O., Samuelsen T.A., Oterhals, Å, Kousoulaki, K., 2018. Optimising cell wall disruption by bead milling of microalgae biomass for release of nutrients for aquafeed and food applications (preliminary results). Book of proceedings, HydroMediT 2018, 3rd International Congress on Applied Ichthyology and Aquatic Environment, 8-11 November 2018, Volos, Greece, pp. 122-126.

Poster presentations

Kokkali M.E., Hovde G., Kleinegris, D.M.M., de Vree J.H., Kousoulaki, K., 2018b. Bead type and agitator configuration effects on the disruption efficiency and protein permeability of the microalgae *Phaeodactylum tricornutum* by bead milling. Poster presentation/Book of abstracts at the ALGAEUROPE 2018 International Conference, 4-6 December 2018, Amsterdam, The Netherlands.

Kousoulaki, K., **Kokkali, M.**, Kleinegris, D.M.M., de Vree, J.H. Haugsgjerd, B.O., Samuelsen, T.A., Oterhals, Å., 2018. Optimizing cell wall disruption of *Tetraselmis chuii* and *Phaeodactylum tricornutum* biomass for release of nutrients and bioactive compounds for aquafeed and food applications. AQUA 2018, Montpellier, France, August 25-29, 2019. Poster presentation/Book of abstracts.

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Appendix

Appendix 1: Raw data for bead milling of *P. tricornutum*, regressors (% Dry matter, Flow rate (kg/h), Tip speed (m/sec)) and depended variables (% Disruption, EPA+DHA release (g/100g Fat), % Aerobic bacteria reduction, Viscosity (mPa), % Soluble protein release, % Fat release).

Bead milling of *P. tricornutum* with small glass beads

Run	% Dry matter	Flow rate (kg/h)	Tip Speed (m/sec)	% Disruption	EPA+DHA (g/100g Fat)
1	15.18	8.65	8.80	55.63	11.40
2	21.43	12.16	8.80	65.71	10.50
3	15.13	7.76	8.80	48.07	11.30
4	21.17	9.09	8.80	54.75	10.30
5	15.05	9.04	11.20	66.48	11.10
6	21.18	7.54	11.20	73.05	10.60
7	14.92	12.37	11.20	65.30	11.50
8	21.56	23.34	11.20	68.98	10.20
9	12.93	10.85	10.00	53.49	11.20
10	23.33	14.40	10.00	68.05	11.40
11	18.12	6.90	10.00	71.64	10.40
12	18.10	15.40	10.00	58.85	10.60
13	18.10	12.36	7.98	61.05	10.40
14	18.22	12.12	12.02	55.06	10.30
15	18.38	13.21	10.00	64.25	10.60
16	18.20	9.85	10.00	62.45	10.50
17	18.28	12.07	10.00	61.25	10.30

Bead milling of *P. tricornutum* with small Zr beads

Run	% Dry matter	Flow rate (kg/h)	Tip Speed (m/sec)	% Disruption	% Aerobic bacteria reduction	Viscosity (mPa)	% Soluble protein release	% Fat release
1	13.30	22.46	7.20	4.93	77.44	23.00	67.32	20.30
2	19.90	9.18	7.20	54.13	43.47	234.00	56.75	13.57
3	13.60	8.97	8.96	42.25	86.95	52.00	61.95	19.12
4	19.90	9.19	8.96	73.99	43.47	244.00	60.60	15.08
5	13.80	8.82	7.20	46.48	76.45	46.00	56.84	18.12
6	20.00	22.48	7.20	63.78	20.00	151.00	43.48	15.00
7	14.90	22.45	8.96	26.06	79.87	12.00	67.95	19.46
8	20.00	22.12	8.96	86.15	1.25	183.00	53.70	14.00
9	11.80	13.69	8.08	15.65	59.75	24.00	56.48	17.80
10	21.50	14.75	8.08	74.50	20.93	157.00	71.09	13.95
11	15.80	5.71	8.08	66.82	69.94	169.00	60.06	14.56
12	16.40	25.81	8.08	37.91	28.35	82.00	56.57	17.07
13	16.50	13.19	6.60	50.71	50.00	106.00	69.39	15.15
14	16.70	13.54	9.56	57.82	61.08	137.00	57.02	16.17
15	16.50	13.13	8.08	59.24	54.55	126.00	59.35	16.36
16	16.50	13.45	8.08	59.72	56.06	119.00	54.68	18.18
17	16.50	13.14	8.08	61.14	57.58	123.00	54.51	16.36

Appendix 2: Row data for bead milling of *T. chuii*, regressors (% Dry matter, Flow rate (kg/h), Tip speed (m/sec)) and depended variables (% Disruption).

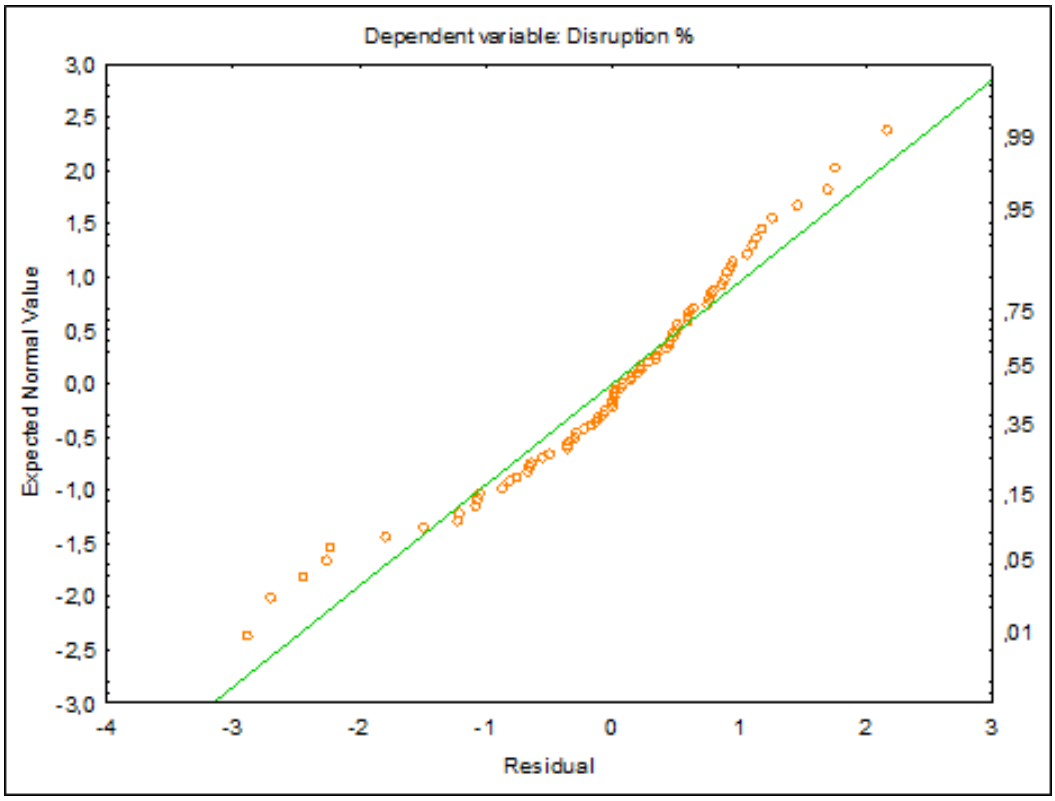
Bead milling of *T. chuii* with small glass beads									
Run	DM %	Flow rate kg/h	Tip speed (m/sec)	% Disruption	Run	DM %	Flow rate kg/h	Tip speed (m/sec)	% Disruption
1	10.0	5.82	8.00	86.86	**39**	17.5	9.54	11.00	93.37
2	10.00	5.80	9.00	74.82	**40**	17.5	9.47	12.00	91.40
3	10.00	5.81	10.00	76.64	**41**	17.5	14.95	8.00	84.77
4	10.00	5.83	11.00	85.40	**42**	17.5	14.72	9.00	85.75
5	10.00	5.82	12.00	85.40	**43**	17.5	14.95	10.00	86.73
6	10.00	9.14	8.00	79.20	**44**	17.5	15.00	11.00	87.47
7	10.0	9.10	9.00	60.95	**45**	17.5	14.62	12.00	85.75
8	10.0	9.14	10.00	84.31	**46**	20.0	6.15	8.00	92.13
9	10.0	9.16	11.00	84.67	**47**	20.0	6.15	9.00	93.17
10	10.0	9.11	12.00	78.10	**48**	20.0	6.55	10.00	94.82
11	10.0	11.11	8.00	79.56	**49**	20.0	6.35	11.00	96.69
12	10.0	10.95	9.00	66.79	**50**	20.0	6.03	12.00	96.89
13	10.0	10.62	10.00	83.21	**51**	20.0	8.94	8.0	94.00
14	10.0	10.71	11.00	82.12	**52**	20.0	9.28	9.0	95.24
15	10.0	10.7	12.00	80.29	**53**	20.0	9.33	10.0	94.20
16	15.0	5.6	8.00	84.57	**54**	20.0	9.74	11.0	95.45
17	15.0	5.9	9.00	87.54	**55**	20.0	8.95	12.0	94.20
18	15.0	5.7	10.00	91.69	**56**	20.0	12.90	8.0	85.51
19	15.0	5.7	11.00	91.39	**57**	20.0	13.94	9.0	85.92
20	15.0	5.7	12.00	91.99	**58**	20.0	13.80	10.0	93.37
21	15.0	8.9	8.00	84.57	**59**	20.0	14.28	11.0	94.20
22	15.0	8.9	9.00	85.46	**60**	20.0	14.27	12.0	93.58
23	15.0	8.9	10.00	90.80	**61**	22.5	4.85	8.0	96.45
24	15.0	8.9	11.00	90.21	**62**	22.5	4.71	9.00	97.69
25	15.0	8.87	12.00	91.10	**63**	22.5	4.75	10.00	98.15
26	15.0	12.00	8.00	77.15	**64**	22.5	3.65	11.00	97.69
27	15.0	12.00	9.00	80.12	**65**	22.5	3.68	12.00	99.38
28	15.0	12.00	10.00	90.80	**66**	22.5	6.12	8.00	96.76
29	15.0	12.00	11.00	87.24	**67**	22.5	5.52	9.00	97.22
30	15.0	12.00	12.00	93.47	**68**	22.5	5.40	10.00	97.84
31	17.5	5.96	8.00	94.35	**69**	22.5	4.08	11.00	97.07
32	17.5	5.94	9.00	93.61	**70**	22.5	4.17	12.00	97.99
33	17.5	5.94	10.00	93.12	**71**	22.5	7.04	8.00	96.76
34	17.5	6.02	11.00	95.58	**72**	22.5	6.61	9.00	97.38
35	17.5	5.80	12.00	94.59	**73**	22.5	5.31	10.00	96.30
36	17.5	9.62	8.00	90.17	**74**	22.5	4.68	11.00	97.53
37	17.5	9.60	9.00	93.37	**75**	22.5	5.08	12.00	95.99
38	17.5	9.45	10.00	83.29					

Appendix 3: Raw data for Pulse electric fileds of of *P. tricornutum* and *T. chuii*, regressors (Solvent, Time (h), pre-treatment) and depended variables (release of Chlorophyl A (C_a), Chlorophyl B (C_B), Total Carotenoids (C_{x+c}), Total Phenolic Compounds (TPC), Trolox Equivalent Antioxidant Capacity (TEAC).

Pre-treatment of *P. tricornutum* with PEF								
Run	Solvent	Time (h)	Pre-Treatment	C_a (mg/g·dw)	C_b (mg/g·dw)	C_{x+c} (mg/g·dw)	TEAC (mM trolox/ gr·dw)	TPC (mg GAE/g·dw)
1	H_2O	4	none	0.000	0.650	0.000	18.927	125.221
2	H_2O	4	PEF1	0.000	0.640	0.000	19.002	125.615
3	H_2O	4	PEF2	2.648	0.939	0.958	52.618	287.438
4	H_2O	4	none	2.640	0.918	0.961	52.587	285.958
5	H_2O	4	PEF1	0.141	0.296	0.000	17.206	160.486
6	H_2O	4	PEF2	0.141	0.290	0.000	17.459	161.048
7	H_2O	24	none	3.755	0.000	1.312	52.458	314.475
8	H_2O	24	PEF1	3.670	0.000	1.320	52.079	315.006
9	H_2O	24	PEF2	0.107	0.164	0.027	32.244	208.093
10	H_2O	24	none	0.106	0.166	0.026	32.231	153.433
11	H_2O	24	PEF1	4.423	0.003	1.496	40.918	323.291
12	H_2O	24	PEF2	4.422	0.000	1.499	40.923	292.140
13	DMSO	4	none	0.231	0.363	0.000	15.680	149.319
14	DMSO	4	PEF1	0.232	0.358	0.000	15.484	167.539
15	DMSO	4	PEF2	2.638	0.005	1.392	51.256	303.895
16	DMSO	4	none	2.640	0.000	1.396	51.454	350.915
17	DMSO	4	PEF1	0.161	0.508	0.000	19.379	191.636
18	DMSO	4	PEF2	0.159	0.507	0.000	19.442	202.215
19	DMSO	24	none	3.936	0.883	1.107	28.996	333.282
20	DMSO	24	PEF1	3.961	0.829	1.129	28.788	281.561
21	DMSO	24	PEF2	0.156	0.512	0.000	15.764	139.327
22	DMSO	24	none	0.155	0.513	0.000	15.792	160.486
23	DMSO	24	PEF1	3.419	0.000	1.271	29.899	310.948
24	DMSO	24	PEF2	3.420	0.000	1.276	29.863	292.728
Pre-treatment of *T. chuii* with PEF								
Run	Solvent	Time (h)	Pre-Treatment	C_a (mg/g·dw)	C_b (mg/g·dw)	C_{x+c} (mg/g·dw)	TEAC (mM trolox/ gr·dw)	TPC (mg GAE/g·dw)
1	H_2O	4	none	0.156	0.105	0.042	37.265	198.101
2	H_2O	4	PEF1	0.152	0.993	0.030	37.015	199.002
3	H_2O	4	PEF2	0.529	0.339	0.149	34.377	242.770
4	H_2O	4	none	0.533	0.316	0.150	34.563	243.655
5	H_2O	4	PEF1	0.194	0.198	0.040	29.112	241.594
6	H_2O	4	PEF2	0.195	0.199	0.027	28.958	242.013
7	H_2O	24	none	0.632	0.386	0.181	48.043	248.060
8	H_2O	24	PEF1	0.622	0.392	0.180	48.156	248.246
9	H_2O	24	PEF2	0.182	0.331	0.025	40.098	260.990
10	H_2O	24	none	0.182	0.331	0.025	40.168	262.165
11	H_2O	24	PEF1	0.686	0.432	0.174	44.846	269.806
12	H_2O	24	PEF2	0.686	0.422	0.177	44.841	270.394
13	DMSO	4	none	0.276	0.791	0.000	21.322	222.787
14	DMSO	4	PEF1	0.275	0.788	0.000	20.927	222.787
15	DMSO	4	PEF2	0.693	0.474	0.174	49.424	264.516
16	DMSO	4	none	0.699	0.468	0.178	49.119	264.516
17	DMSO	4	PEF1	0.018	0.584	0.000	44.682	198.101
18	DMSO	4	PEF2	0.016	0.584	0.000	44.569	202.215
19	DMSO	24	none	0.711	0.170	0.402	52.071	215.734
20	DMSO	24	PEF1	0.707	0.180	0.394	51.865	218.085
21	DMSO	24	PEF2	0.341	0.424	0.101	31.563	229.252
22	DMSO	24	none	0.338	0.425	0.100	31.566	229.252
23	DMSO	24	PEF1	0.698	0.000	0.468	35.021	237.480
24	DMSO	24	PEF2	0.727	0.000	0.494	34.921	237.480

Where:		Field strength [kV/cm]	Pulses	Voltage [kV]	Energy [J]/ pulse	Specific energy [kJ/kg]
	PEF1	1	400	10	50	100
	PEF2	3	45	30	450	101.3
	none	0	0	0	0	0

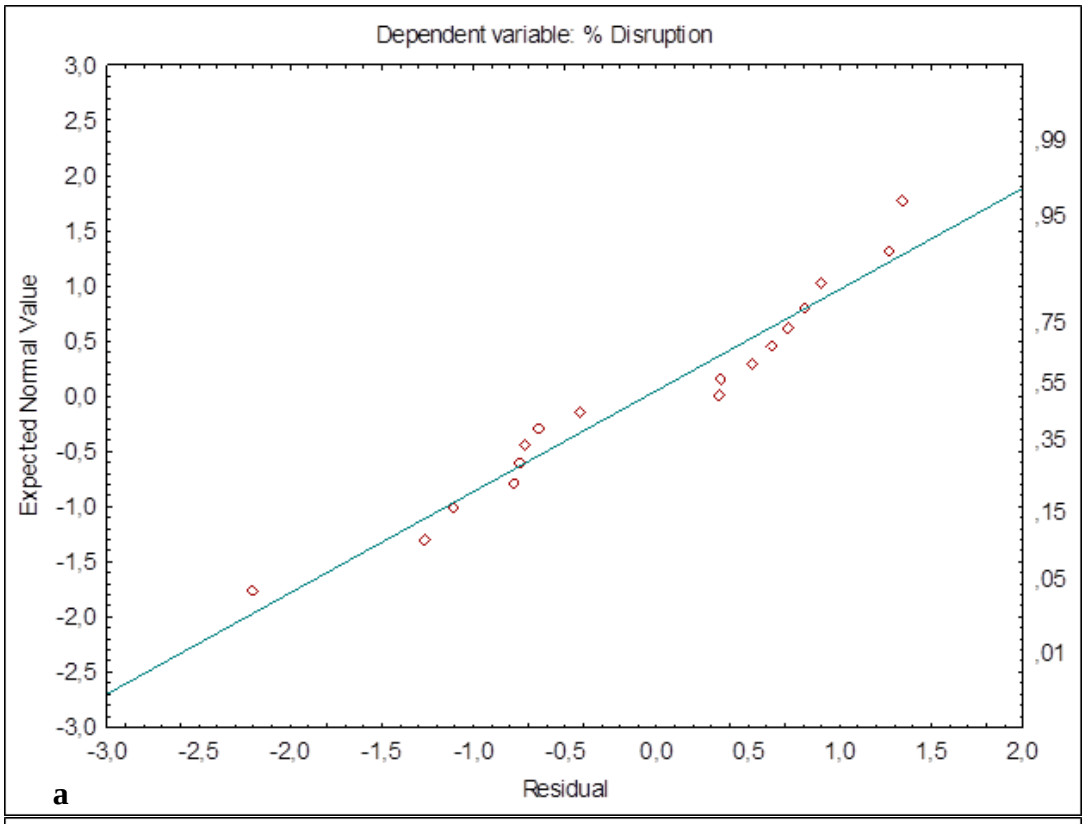
Appendix 4: Probability plot of the residuals normally distributed after removing two outlier values for bead milling of *T. chuii.*



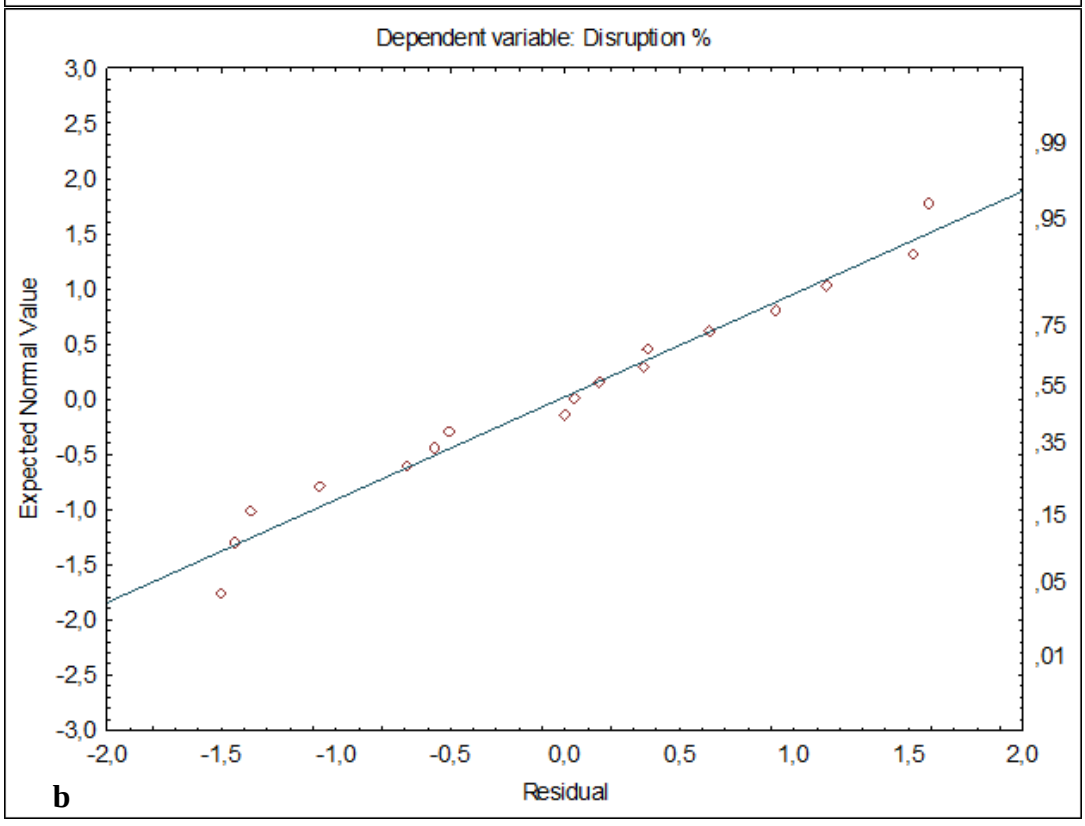
Appendix 5: Summary of stepwise regression for correlation of % Disruption of *T. chuii* after bead milling with small glass beads.
Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: %Disruption Backward only P to enter: ,05, P to remove: ,05						
Effect	Steps	Degree of freedom	F to remove	P to remove	F to enter	P to enter	Effect status
Dry matter %	Step Number 1	1	5.2913	0.024554			In
Dry matter %^2		1	1.9194	0.170521			In
Flow rate (kg/h)		1	0.4584	0.500695			In
Flow rate (kg/h)^2		1	2.1716	0.145260			In
Tip speed (m/sec)		1	0.6672	0.416914			In
Tip speed (m/sec)^2		1	0.6680	0.416653			In
Dry matter %*Flow rate (kg/h)		1	0.0047	0.945571			Removed
Dry matter %*Tip speed (m/sec)		1	0.1398	0.709698			In
Flow rate (kg/h)*Tip speed (m/sec)		1	3.3413	0.072013			In
Dry matter %	Step Number 2	1	7.3936	0.008300			In
Dry matter %^2		1	2.2019	0.142464			In
Flow rate (kg/h)		1	0.5625	0.455841			In
Flow rate (kg/h)^2		1	2.3065	0.133473			In
Tip speed (m/sec)		1	0.6761	0.413812			In
Tip speed (m/sec)^2		1	0.6760	0.413837			In
Flow rate (kg/h)*Tip speed (m/sec)		1	3.4104	0.069139			In
Dry matter %*Tip speed (m/sec)		1	0.1388	0.710631			Removed
Dry matter %*Flow rate (kg/h)		1			0.004696	0.945571	Out
Dry matter %	Step Number 3	1	10.6082	0.001748			In
Dry matter %^2		1	2.1753	0.144794			In
Flow rate (kg/h)		1	0.5957	0.442857			In
Flow rate (kg/h)^2		1	2.6455	0.108405			In
Tip speed (m/sec)		1	0.5903	0.444907			Removed
Tip speed (m/sec)^2		1	0.7425	0.391830			In
Flow rate (kg/h)*Tip speed (m/sec)		1	4.1260	0.046084			In
Dry matter %*Tip speed (m/sec)		1			0.138805	0.710631	Out
Dry matter %*Flow rate (kg/h)		1			0.001729	0.966955	Out
Dry matter %	Step Number 4	1	10.9862	0.001456			In
Dry matter %^2		1	2.3324	0.131210			In
Flow rate (kg/h)		1	0.8868	0.349581			In
Flow rate (kg/h)^2		1	2.5348	0.115867			In
Flow rate (kg/h)*Tip speed (m/sec)		1	5.2553	0.024890			In
Tip speed (m/sec)^2		1	0.4228	0.517652			Removed
Tip speed (m/sec)		1			0.590346	0.444907	Out
Dry matter %*Tip speed (m/sec)		1			0.045913	0.830966	Out
Dry matter %*Flow rate (kg/h)		1			0.002055	0.963972	Out
Dry matter %	Step Number 5	1	11.2836	0.001261			In
Dry matter %^2		1	2.4466	0.122223			In
Flow rate (kg/h)		1	0.4688	0.495760			Removed
Flow rate (kg/h)^2		1	2.9802	0.088639			In
Flow rate (kg/h)*Tip speed (m/sec)		1	21.1116	0.000018			In
Tip speed (m/sec)^2		1			0.422840	0.517652	Out
Tip speed (m/sec)		1			0.269151	0.605538	Out
Dry matter %*Tip speed (m/sec)		1			0.451118	0.504015	Out
Dry matter %*Flow rate (kg/h)		1			0.005449	0.941367	Out
Dry matter %	Step Number 6	1	10.8969	0.001501			In
Dry matter %^2		1	2.0639	0.155151			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	20.9695	0.000019			In
Flow rate (kg/h)^2		1	41.9265	0.000000			In
Flow rate (kg/h)		1			0.468819	0.495760	Out
Tip speed (m/sec)^2		1			0.001037	0.974399	Out
Tip speed (m/sec)		1			0.018760	0.891445	Out
Dry matter %*Tip speed (m/sec)		1			0.002710	0.958626	Out
Dry matter %*Flow rate (kg/h)		1			0.109313	0.741901	Out
Dry matter %	Step Number 7	1	262.4642	0.000000			In
Flow rate (kg/h)^2		1	39.5730	0.000000			In
Flow rate (kg/h)*Tip speed (m/sec)		1	21.3088	0.000016			In
Dry matter %^2		1			2.063944	0.155151	Out
Flow rate (kg/h)		1			0.069493	0.792830	Out
Tip speed (m/sec)^2		1			0.086738	0.769213	Out
Tip speed (m/sec)		1			0.034975	0.852174	Out
Dry matter %*Tip speed (m/sec)		1			0.102351	0.749953	Out
Dry matter %*Flow rate (kg/h)		1			0.129570	0.719932	Out

Appendix 6: Probability plot of the residuals normally distributed following *a) bead milling with Zr beads and b) bead milling with glass beads*



a



b

Appendix 7: Summary of stepwise regression for correlation of % Disruption of *P. tricornutum,* after bead milling with small glass beads.
Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: Disruption % Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number 1	1	1.28666	0.29401			In
Dry matter %^2		1	0.34608	0.574824			Removed
Flow rate (kg/h)		1	3.62855	0.098497			In
Flow rate (kg/h)^2		1	6.05761	0.043388			In
Tip speed (m/sec)		1	5.44356	0.052371			In
Tip speed (m/sec)^2		1	0.82681	0.393426			In
Dry matter %*Flow rate (kg/h)		1	0.42743	0.534131			In
Dry matter %*Tip speed (m/sec)		1	1.3658	0.280789			In
Flow rate (kg/h)*Tip speed (m/sec)		1	8.95519	0.020154			In
Dry matter %	Step Number 2	1	1.08673	0.327672			In
Flow rate (kg/h)*Tip speed (m/sec)		1	9.49388	0.015089			In
Flow rate (kg/h)		1	4.71128	0.061766			In
Flow rate (kg/h)^2		1	7.3462	0.026642			In
Tip speed (m/sec)		1	5.55164	0.046232			In
Tip speed (m/sec)^2		1	0.70606	0.425154			In
Dry matter %*Flow rate (kg/h)		1	0.20354	0.663853			Removed
Dry matter %*Tip speed (m/sec)		1	1.34519	0.279563			In
Dry matter %^2		1			0.346076	0.574824	Out
Dry matter %	Step Number 3	1	1.71919	0.222261			In
Flow rate (kg/h)*Tip speed (m/sec)		1	10.3912	0.01043			In
Flow rate (kg/h)		1	6.46272	0.031597			In
Flow rate (kg/h)^2		1	10.0409	0.011389			In
Tip speed (m/sec)		1	5.97591	0.037086			In
Tip speed (m/sec)^2		1	0.87165	0.374869			Removed
Dry matter %*Tip speed (m/sec)		1	1.37998	0.270248			In
Dry matter %*Flow rate (kg/h)		1			0.203544	0.663853	Out
Dry matter %^2		1			0.113687	0.744661	Out
Dry matter %	Step Number 4	1	1.79504	0.209963			In
Flow rate (kg/h)*Tip speed (m/sec)		1	11.1168	0.007564			In
Flow rate (kg/h)		1	6.90623	0.02525			In
Flow rate (kg/h)^2		1	10.7064	0.008401			In
Tip speed (m/sec)		1	9.24757	0.012442			In
Dry matter %*Tip speed (m/sec)		1	1.45107	0.256092			Removed
Tip speed (m/sec)^2		1			0.871648	0.374869	Out
Dry matter %*Flow rate (kg/h)		1			0.301855	0.596089	Out
Dry matter %^2		1			0.015749	0.902891	Out
Dry matter %	Step Number 5	1	2.32785	0.155301			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	10.6259	0.007603			In
Flow rate (kg/h)		1	7.99324	0.016453			In
Flow rate (kg/h)^2		1	8.9014	0.012441			In
Tip speed (m/sec)		1	13.0195	0.00411			In
Dry matter %*Tip speed (m/sec)		1			1.451066	0.256092	Out
Tip speed (m/sec)^2		1			0.890278	0.36764	Out
Dry matter %*Flow rate (kg/h)		1			0.183651	0.677338	Out
Dry matter %^2		1			0.003505	0.953956	Out
Tip speed (m/sec)	Step Number 6	1	17.5092	0.001267			In
Flow rate (kg/h)*Tip speed (m/sec)		1	15.2796	0.002076			In
Flow rate (kg/h)		1	11.5586	0.005272			In
Flow rate (kg/h)^2		1	12.5818	0.004018			In
Dry matter %		1			2.327854	0.155301	Out
Dry matter %*Tip speed (m/sec)		1			1.939181	0.191265	Out
Tip speed (m/sec)^2		1			0.73107	0.410773	Out
Dry matter %*Flow rate (kg/h)		1			2.553773	0.138337	Out
Dry matter %^2		1			2.279967	0.159233	Out

Appendix 8: Summary of stepwise regression for correlation of EPA+DHA release from *P. tricornutum* after bead milling with small glass beads. Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Backward only P to enter: .05, P to remove: .05							
Effect	**Steps**		**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number	1	1	6.05548	0.043415			In
Dry matter %^2			1	4.79783	0.064641			In
Flow rate (kg/h)			1	0.19931	0.668764			In
Flow rate (kg/h)^2			1	1.49659	0.260776			In
Tip speed (m/sec)			1	0.00022	0.988476			Removed
Tip speed (m/sec)^2			1	0.08946	0.773554			In
Dry matter %*Flow rate (kg/h)			1	0.94618	0.363099			In
Dry matter %*Tip speed (m/sec)			1	0.26737	0.621035			In
Flow rate (kg/h)*Tip speed (m/sec)			1	0.00984	0.923751			In
Dry matter %	Step Number	2	1	8.74706	0.018214			In
Dry matter %^2			1	5.90142	0.041248			In
Flow rate (kg/h)			1	0.22942	0.644776			In
Flow rate (kg/h)^2			1	1.81236	0.215124			In
Flow rate (kg/h)*Tip speed (m/sec)			1	0.01295	0.912185			Removed
Tip speed (m/sec)^2			1	0.30315	0.596946			In
Dry matter %*Flow rate (kg/h)			1	1.10734	0.323406			In
Dry matter %*Tip speed (m/sec)			1	0.39451	0.547447			In
Tip speed (m/sec)			1			0.000224	0.988476	Out
Dry matter %	Step Number	3	1	10.20645	0.010923			In
Dry matter %^2			1	6.61776	0.030064			In
Flow rate (kg/h)			1	0.38733	0.549147			In
Flow rate (kg/h)^2			1	2.46217	0.151064			In
Dry matter %*Tip speed (m/sec)			1	0.45931	0.514991			In
Tip speed (m/sec)^2			1	0.38485	0.550402			Removed
Dry matter %*Flow rate (kg/h)			1	1.40573	0.266124			In
Flow rate (kg/h)*Tip speed (m/sec)			1			0.012955	0.912185	Out
Tip speed (m/sec)			1			0.001959	0.965778	Out
Dry matter %	Step Number	4	1	14.75668	0.003257			In
Dry matter %^2			1	7.02990	0.024255			In
Flow rate (kg/h)			1	0.82958	0.383824			In
Flow rate (kg/h)^2			1	2.24658	0.164799			In
Dry matter %*Tip speed (m/sec)			1	0.23304	0.639674			Removed
Dry matter %*Flow rate (kg/h)			1	1.73856	0.216721			In
Tip speed (m/sec)^2			1			0.384850	0.550402	Out
Flow rate (kg/h)*Tip speed (m/sec)			1			0.056845	0.816894	Out
Tip speed (m/sec)			1			0.274701	0.612857	Out
Dry matter %	Step Number	5	1	15.61263	0.002268			In
Dry matter %^2			1	7.97575	0.016545			In
Flow rate (kg/h)			1	0.74851	0.405421			Removed
Flow rate (kg/h)^2			1	2.16815	0.168922			In
Dry matter %*Flow rate (kg/h)			1	1.63067	0.227908			In
Dry matter %*Tip speed (m/sec)			1			0.233038	0.639674	Out
Tip speed (m/sec)^2			1			0.152491	0.704356	Out
Flow rate (kg/h)*Tip speed (m/sec)			1			0.173771	0.685589	Out
Tip speed (m/sec)			1			0.170367	0.688496	Out
Dry matter %	Step Number	6	1	16.05266	0.001741			In
Dry matter %^2			1	12.02116	0.004655			In
Dry matter %*Flow rate (kg/h)			1	1.28353	0.279373			Removed
Flow rate (kg/h)^2			1	1.65277	0.222838			In
Flow rate (kg/h)			1			0.748511	0.405421	Out
Dry matter %*Tip speed (m/sec)			1			0.101350	0.756178	Out
Tip speed (m/sec)^2			1			0.035041	0.854919	Out
Flow rate (kg/h)*Tip speed (m/sec)			1			0.001935	0.965702	Out
Tip speed (m/sec)			1			0.042940	0.839625	Out
Dry matter %	Step Number	7	1	15.78867	0.001590			In
Dry matter %^2			1	14.10419	0.002402			In
Flow rate (kg/h)^2			1	1.00095	0.335339			Removed
Dry matter %*Flow rate (kg/h)			1			1.283531	0.279373	Out
Flow rate (kg/h)			1			0.355774	0.561946	Out
Dry matter %*Tip speed (m/sec)			1			0.000128	0.991167	Out
Tip speed (m/sec)^2			1			0.002660	0.959713	Out
Flow rate (kg/h)*Tip speed (m/sec)			1			0.108254	0.747812	Out
Tip speed (m/sec)			1			0.001219	0.972725	Out
Dry matter %	Step Number	8	1	15.23274	0.001593			In
Dry matter %^2			1	13.41626	0.002559			In
Flow rate (kg/h)^2			1			1.000953	0.335339	Out
Dry matter %*Flow rate (kg/h)			1			0.622295	0.444345	Out
Flow rate (kg/h)			1			0.692669	0.420285	Out
Dry matter %*Tip speed (m/sec)			1			0.089710	0.769281	Out
Tip speed (m/sec)^2			1			0.110983	0.744339	Out
Flow rate (kg/h)*Tip speed (m/sec)			1			0.724768	0.409993	Out
Tip speed (m/sec)			1			0.099398	0.757557	Out

Appendix 9: Summary of stepwise regression for correlation of % Disruption of *P. tricornutum* after bead milling with Zr beads.

Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: Disruption % Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
DM %	Step Number 1	1	1.2777	0.295571			In
DM %^2		1	83.278	0.000039			In
Flow Rate (kg/h)		1	50.2925	0.000195			In
Flow Rate (kg/h)^2		1	4.7161	0.066455			In
Tip speed (m/sec)		1	1.6305	0.242357			In
Tip speed (m/sec)^2		1	14.7374	0.006383			In
DM %*Flow Rate (kg/h)		1	206.208	0.000002			In
DM %*Tip speed (m/sec)		1	54.0212	0.000156			In
Flow Rate (kg/h)*Tip speed (m/sec)		1	0.236	0.641949			Removed
DM %	Step Number 2	1	1.4641	0.260817			Removed
DM %^2		1	95.7917	0.00001			In
Flow Rate (kg/h)		1	123.1633	0.000004			In
Flow Rate (kg/h)^2		1	5.336	0.049691			In
Tip speed (m/sec)		1	2.0224	0.192793			In
Tip speed (m/sec)^2		1	16.4288	0.003668			In
DM %*Flow Rate (kg/h)		1	231.2257	0			In
DM %*Tip speed (m/sec)		1	60.942	0.000052			In
Flow Rate (kg/h)*Tip speed (m/sec)		1			0.235998	0.641949	Out
DM %*Tip speed (m/sec)	Step Number 3	1	123.5662	0.000001			In
DM %^2		1	139.8565	0.000001			In
Flow Rate (kg/h)		1	134.1711	0.000001			In
Flow Rate (kg/h)^2		1	4.3618	0.066346			In
Tip speed (m/sec)		1	1.0208	0.338722			Removed
Tip speed (m/sec)^2		1	14.5187	0.004151			In
DM %*Flow Rate (kg/h)		1	237.2257	0			In
DM %		1			1.464095	0.260817	Out
Flow Rate (kg/h)*Tip speed (m/sec)		1			0.273128	0.615402	Out
DM %*Tip speed (m/sec)	Step Number 4	1	125.5521	0.000001			In
DM %^2		1	141.422	0			In
Flow Rate (kg/h)		1	137.9073	0			In
Flow Rate (kg/h)^2		1	3.7474	0.081643			Removed
DM %*Flow Rate (kg/h)		1	235.9833	0			In
Tip speed (m/sec)^2		1	97.6087	0.000002			In
Tip speed (m/sec)		1			1.02079	0.338722	Out
DM %		1			0.462613	0.513519	Out
Flow Rate (kg/h)*Tip speed (m/sec)		1			0.419238	0.533483	Out
DM %*Tip speed (m/sec)	Step Number 5	1	97.471	0.000001			In
DM %^2		1	110.4076	0			In
Flow Rate (kg/h)		1	232.0536	0			In
Tip speed (m/sec)^2		1	75.1121	0.000003			In
DM %*Flow Rate (kg/h)		1	186.0266	0			In
Flow Rate (kg/h)^2		1			3.747433	0.081643	Out
Tip speed (m/sec)		1			0.310014	0.589914	Out
DM %		1			0.212436	0.654726	Out
Flow Rate (kg/h)*Tip speed (m/sec)		1			0.372229	0.555402	Out

Appendix 10: Summary of stepwise regression for correlation of % Aerobic bacteria reduction from *P. tricornutum biomass* after bead milling with Zr beads.
Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: % Aerobic bacteria reduction Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number 1	1	164005.00	0.2411			In
Dry matter %^2		1	0.47	0.5148			In
Flow rate (kg/h)		1	0.89	0.3767			In
Flow rate (kg/h)^2		1	0.07	0.7941			In
Tip speed (m/sec)		1	0.03	0.8700			Removed
Tip speed (m/sec)^2		1	0.45	0.5221			In
Dry matter %*Flow rate (kg/h)		1	368962.00	0.0962			In
Dry matter %*Tip speed (m/sec)		1	121607.00	0.3066			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.17	0.6952			In
Dry matter %	Step Number 2	1	242565.00	0.1580			In
Dry matter %^2		1	0.63	0.4486			In
Flow rate (kg/h)		1	115776.00	0.3133			In
Flow rate (kg/h)^2		1	0.06	0.8057			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	0.21	0.6554			In
Tip speed (m/sec)^2		1	226027.00	0.1711			In
Dry matter %*Flow rate (kg/h)		1	423385.00	0.0736			In
Dry matter %*Tip speed (m/sec)		1	164399.00	0.2357			In
Tip speed (m/sec)		1			0.0288	0.8700	Out
Dry matter %	Step Number 3	1	280077.00	0.1285			In
Dry matter %^2		1	0.85	0.3817			In
Flow rate (kg/h)		1	191635.00	0.1996			In
Dry matter %*Tip speed (m/sec)		1	178287.00	0.2146			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.24	0.6329			Removed
Tip speed (m/sec)^2		1	246610.00	0.1508			In
Dry matter %*Flow rate (kg/h)		1	466373.00	0.0591			In
Flow rate (kg/h)^2		1			0.0647	0.8057	Out
Tip speed (m/sec)		1			0.0137	0.9097	Out
Dry matter %	Step Number 4	1	305383.00	0.1111			In
Dry matter %^2		1	0.81	0.3896			Removed
Flow rate (kg/h)		1	347619.00	0.0918			In
Dry matter %*Tip speed (m/sec)		1	216358.00	0.1721			In
Dry matter %*Flow rate (kg/h)		1	531325.00	0.0439			In
Tip speed (m/sec)^2		1	241230.00	0.1514			In
Flow rate (kg/h)*Tip speed (m/sec)		1			0.2443	0.6329	Out
Flow rate (kg/h)^2		1			0.0754	0.7898	Out
Tip speed (m/sec)		1			0.0332	0.8594	Out
Dry matter %	Step Number 5	1	238772.00	0.1506			Removed
Tip speed (m/sec)^2		1	301287.00	0.1105			In
Flow rate (kg/h)		1	388008.00	0.0746			In
Dry matter %*Tip speed (m/sec)		1	268914.00	0.1293			In
Dry matter %*Flow rate (kg/h)		1	584843.00	0.0341			In
Dry matter %^2		1			0.8088	0.3896	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.1483	0.7082	Out
Flow rate (kg/h)^2		1			0.2107	0.6560	Out
Tip speed (m/sec)		1			0.1107	0.7463	Out
Dry matter %*Flow rate (kg/h)	Step Number 6	1	357888.00	0.0829			In
Tip speed (m/sec)^2		1	0.72	0.4136			In
Flow rate (kg/h)		1	205465.00	0.1773			In
Dry matter %*Tip speed (m/sec)		1	0.29	0.5971			Removed
Dry matter %		1			2387719.0000	0.1506	Out
Dry matter %^2		1			0.0852	0.7757	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.2933	0.5989	Out
Flow rate (kg/h)^2		1			0.1080	0.7486	Out
Tip speed (m/sec)		1			0.6215	0.4472	Out
Dry matter %*Flow rate (kg/h)	Step Number 7	1	4853824.00	0.0000			In
Tip speed (m/sec)^2		1	0.59	0.4563			Removed
Flow rate (kg/h)		1	2553042.00	0.0002			In
Dry matter %*Tip speed (m/sec)		1			0.2948	0.5971	Out
Dry matter %		1			0.0240	0.8794	Out
Dry matter %^2		1			0.1725	0.6852	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.3012	0.5932	Out
Flow rate (kg/h)^2		1			0.1344	0.7203	Out
Tip speed (m/sec)		1			0.6993	0.4193	Out
Dry matter %*Flow rate (kg/h)	Step Number 8	1	4953419.00	0.0000			In
Flow rate (kg/h)		1	2595830.00	0.0002			In
Tip speed (m/sec)^2		1			0.5895	0.4563	Out
Dry matter %*Tip speed (m/sec)		1			0.1381	0.7161	Out
Dry matter %		1			0.0330	0.8587	Out
Dry matter %^2		1			0.2126	0.6524	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.2767	0.6078	Out
Flow rate (kg/h)^2		1			0.1230	0.7314	Out
Tip speed (m/sec)		1			0.5182	0.4843	Out

Appendix 11: Summary of stepwise regression for correlation of Viscosity alterations of *P. tricornutum* biomass after bead milling with Zr beads. Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: Viscosity 10^{-1} (mPa) Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number 1	1	0.3232	0.5874			In
Dry matter %^2		1	0.5801	0.4711			In
Flow rate (kg/h)		1	0.0011	0.9747			Removed
Flow rate (kg/h)^2		1	0.9472	0.3629			In
Tip speed (m/sec)		1	0.0157	0.9038			In
Tip speed (m/sec)^2		1	0.0011	0.9743			In
Dry matter %*Flow rate (kg/h)		1	0.1964	0.6710			In
Dry matter %*Tip speed (m/sec)		1	0.5547	0.4806			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.3740	0.5602			In
Dry matter %	Step Number 2	1	0.3764	0.5566			In
Dry matter %^2		1	0.6759	0.4348			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.9694	0.3537			In
Flow rate (kg/h)^2		1	141133.00	0.2689			In
Tip speed (m/sec)		1	0.0212	0.8878			In
Tip speed (m/sec)^2		1	0.0010	0.9757			Removed
Dry matter %*Flow rate (kg/h)		1	0.2467	0.6328			In
Dry matter %*Tip speed (m/sec)		1	0.6338	0.4489			In
Flow rate (kg/h)		1			0.0011	0.9747	Out
Dry matter %	Step Number 3	1	0.4260	0.5303			In
Dry matter %^2		1	0.7956	0.3956			In
Flow rate (kg/h)*Tip speed (m/sec)		1	111218.00	0.3191			In
Flow rate (kg/h)^2		1	168188.00	0.2269			In
Tip speed (m/sec)		1	0.2669	0.6179			Removed
Dry matter %*Tip speed (m/sec)		1	0.7120	0.4207			In
Dry matter %*Flow rate (kg/h)		1	0.2808	0.6090			In
Tip speed (m/sec)^2		1			0.0010	0.9757	Out
Flow rate (kg/h)		1			0.0009	0.9762	Out
Dry matter %	Step Number 4	1	169950.00	0.2216			In
Dry matter %^2		1	0.7760	0.3990			In
Flow rate (kg/h)*Tip speed (m/sec)		1	132784.00	0.2760			In
Flow rate (kg/h)^2		1	207988.00	0.1798			In
Dry matter %*Flow rate (kg/h)		1	0.3129	0.5882			Removed
Dry matter %*Tip speed (m/sec)		1	170756.00	0.2206			In
Tip speed (m/sec)		1			0.2669	0.6179	Out
Tip speed (m/sec)^2		1			0.2435	0.6335	Out
Flow rate (kg/h)		1			0.0324	0.8611	Out
Dry matter %	Step Number 5	1	159769.00	0.2324			In
Dry matter %^2		1	106243.00	0.3248			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	435082.00	0.0611			In
Flow rate (kg/h)^2		1	191255.00	0.1941			In
Dry matter %*Tip speed (m/sec)		1	403235.00	0.0698			In
Dry matter %*Flow rate (kg/h)		1			0.3129	0.5882	Out
Tip speed (m/sec)		1			0.2975	0.5974	Out
Tip speed (m/sec)^2		1			0.2843	0.6055	Out
Flow rate (kg/h)		1			0.0006	0.9809	Out
Dry matter %	Step Number 6	1	132781.00	0.2716			Removed
Dry matter %*Tip speed (m/sec)		1	451435.00	0.0551			In
Flow rate (kg/h)*Tip speed (m/sec)		1	476513.00	0.0496			In
Flow rate (kg/h)^2		1	219078.00	0.1646			In
Dry matter %^2		1			1062433.0000	0.3248	Out
Dry matter %*Flow rate (kg/h)		1			0.5440	0.4762	Out
Tip speed (m/sec)		1			0.2034	0.6607	Out
Tip speed (m/sec)^2		1			0.1229	0.7326	Out
Flow rate (kg/h)		1			0.0643	0.8046	Out
Flow rate (kg/h)^2	Step Number 7	1	1108394.00	0.0054			In
Dry matter %*Tip speed (m/sec)		1	8095589.00	0.0000			In
Flow rate (kg/h)*Tip speed (m/sec)		1	1948770.00	0.0007			In
Dry matter %		1			1327814.0000	0.2716	Out
Dry matter %^2		1			0.7615	0.4000	Out
Dry matter %*Flow rate (kg/h)		1			0.2975	0.5955	Out
Tip speed (m/sec)		1			1539447.0000	0.2384	Out
Tip speed (m/sec)^2		1			1473048.0000	0.2482	Out
Flow rate (kg/h)		1			0.5310	0.4802	Out

Appendix 12: Summary of stepwise regression for correlation of % Fat release, after bead milling with Zr beads.

Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: % Fat release Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number 1	1	0.0178	0.8976			In
Dry matter %^2		1	0.0716	0.7968			In
Flow rate (kg/h)		1	203110	0.1971			In
Flow rate (kg/h)^2		1	0.2950	0.6039			In
Tip speed (m/sec)		1	0.2579	0.6272			In
Tip speed (m/sec)^2		1	0.1183	0.7410			In
Dry matter %*Flow rate (kg/h)		1	0.8007	0.4006			In
Dry matter %*Tip speed (m/sec)		1	0.0132	0.9119			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	0.6409	0.4497			In
Dry matter %	Step Number 2	1	0.0068	0.9361			Removed
Dry matter %^2		1	0.0898	0.7721			In
Flow rate (kg/h)		1	231762	0.1664			In
Flow rate (kg/h)^2		1	0.3396	0.5761			In
Tip speed (m/sec)		1	0.2804	0.6108			In
Tip speed (m/sec)^2		1	0.1402	0.7178			In
Dry matter %*Flow rate (kg/h)		1	0.8993	0.3707			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.7564	0.4098			In
Dry matter %*Tip speed (m/sec)		1			0.0132	0.9119	Out
Flow rate (kg/h)*Tip speed (m/sec)	Step Number 3	1	0.8436	0.3823			In
Dry matter %^2		1	100644	0.3420			In
Flow rate (kg/h)		1	272862	0.1330			In
Flow rate (kg/h)^2		1	0.3775	0.5542			In
Tip speed (m/sec)		1	0.3123	0.5899			In
Tip speed (m/sec)^2		1	0.1502	0.7074			Removed
Dry matter %*Flow rate (kg/h)		1	100511	0.3423			In
Dry matter %		1			0.0068	0.9361	Out
Dry matter %*Tip speed (m/sec)		1			0.0016	0.9695	Out
Flow rate (kg/h)*Tip speed (m/sec)	Step Number 4	1	0.9301	0.3576			In
Dry matter %^2		1	107291	0.3247			In
Flow rate (kg/h)		1	290627	0.1191			In
Flow rate (kg/h)^2		1	0.3292	0.5788			Removed
Tip speed (m/sec)		1	141902	0.2611			In
Dry matter %*Flow rate (kg/h)		1	113078	0.3126			In
Tip speed (m/sec)^2		1			0.1502	0.7074	Out
Dry matter %		1			0.0003	0.9864	Out
Dry matter %*Tip speed (m/sec)		1			0.0152	0.9047	Out
Flow rate (kg/h)*Tip speed (m/sec)	Step Number 5	1	0.9701	0.3458			Removed
Dry matter %^2		1	101450	0.3355			In
Flow rate (kg/h)		1	276927	0.1243			In
Dry matter %*Flow rate (kg/h)		1	133631	0.2722			In
Tip speed (m/sec)		1	149908	0.2464			In
Flow rate (kg/h)^2		1			0.3292	0.5788	Out
Tip speed (m/sec)^2		1			0.0788	0.7847	Out
Dry matter %		1			0.0144	0.9069	Out
Dry matter %*Tip speed (m/sec)		1			0.0396	0.8462	Out
Tip speed (m/sec)	Step Number 6	1	0.7854	0.3929			Removed
Dry matter %^2		1	0.8792	0.3669			In
Flow rate (kg/h)		1	244032	0.1442			In
Dry matter %*Flow rate (kg/h)		1	157847	0.2329			In
Flow rate (kg/h)*Tip speed (m/sec)		1			0.9701	0.3458	Out
Flow rate (kg/h)^2		1			0.3120	0.5877	Out
Tip speed (m/sec)^2		1			0.0871	0.7735	Out
Dry matter %		1			0.0561	0.8171	Out
Dry matter %*Tip speed (m/sec)		1			0.1154	0.7405	Out
Dry matter %*Flow rate (kg/h)	Step Number 7	1	146476	0.2477			In
Dry matter %^2		1	0.9833	0.3395			Removed
Flow rate (kg/h)		1	231050	0.1524			In
Tip speed (m/sec)		1			0.7854	0.3929	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.2443	0.6301	Out
Flow rate (kg/h)^2		1			0.3440	0.5684	Out
Tip speed (m/sec)^2		1			0.7526	0.4027	Out
Dry matter %		1			0.0351	0.8545	Out
Dry matter %*Tip speed (m/sec)		1			0.6439	0.4379	Out
Dry matter %*Flow rate (kg/h)	Step Number 8	1	4048893	0.0000			In
Flow rate (kg/h)		1	4252110	0.0000			In
Dry matter %^2		1			1	0.3395	Out
Tip speed (m/sec)		1			0.8814	0.3649	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.3199	0.5813	Out
Flow rate (kg/h)^2		1			0.2228	0.6447	Out
Tip speed (m/sec)^2		1			0.8474	0.3741	Out
Dry matter %		1			1020940.0000	0.3307	Out
Dry matter %*Tip speed (m/sec)		1			0.0835	0.7772	Out

Appendix 13: Summary of stepwise regression for correlation of % Soluble protein release, after bead milling with Zr beads.

Parameters (%Dry matter, Flow rate (kg/h), Tip Speed (m/sec)).

	Summary of stepwise regression; variable: % Soluble protein release Backward only P to enter: .05, P to remove: .05						
Effect	**Steps**	**Degree of Flow**	**F to remove**	**P to remove**	**F to enter**	**P to enter**	**Effect status**
Dry matter %	Step Number 1	1	0.1851	0.6799			In
Dry matter %^2		1	0.4650	0.5172			In
Flow rate (kg/h)		1	0	0.7289			In
Flow rate (kg/h)^2		1	0.0042	0.9502			Removed
Tip speed (m/sec)		1	0.5793	0.4715			In
Tip speed (m/sec)^2		1	0.3913	0.5515			In
Dry matter %*Flow rate (kg/h)		1	3203374	0.1166			In
Dry matter %*Tip speed (m/sec)		1	0.0668	0.8035			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.3915	0.5514			In
Dry matter %	Step Number 2	1	0.2282	0.6457			In
Dry matter %^2		1	0.5960	0.4623			In
Flow rate (kg/h)		1	0	0.7010			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.4525	0.5201			In
Tip speed (m/sec)		1	0.7267	0.4187			In
Tip speed (m/sec)^2		1	0.5006	0.4993			In
Dry matter %*Flow rate (kg/h)		1	3691295	0.0909			In
Dry matter %*Tip speed (m/sec)		1	0.0755	0.7904			Removed
Flow rate (kg/h)^2		1			0.0042	0.9502	Out
Dry matter %	Step Number 3	1	0.1735	0.6868			Removed
Dry matter %^2		1	1	0.4185			In
Flow rate (kg/h)		1	0	0.6824			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.5463	0.4787			In
Tip speed (m/sec)		1	0.7262	0.4162			In
Tip speed (m/sec)^2		1	0.5812	0.4653			In
Dry matter %*Flow rate (kg/h)		1	4411050	0.0651			In
Dry matter %*Tip speed (m/sec)		1			0.0755	0.7904	Out
Flow rate (kg/h)^2		1			0.0040	0.9512	Out
Dry matter %*Flow rate (kg/h)	Step Number 4	1	5216383	0.0455			In
Dry matter %^2		1	3788186	0.0802			In
Flow rate (kg/h)		1	0	0.6055			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	0.5234	0.4860			In
Tip speed (m/sec)		1	1	0.4361			In
Tip speed (m/sec)^2		1	1	0.4891			In
Dry matter %		1			0.1735	0.6868	Out
Dry matter %*Tip speed (m/sec)		1			0.0033	0.9552	Out
Flow rate (kg/h)^2		1			0.0260	0.8755	Out
Dry matter %*Flow rate (kg/h)	Step Number 5	1	5583495	0.0376			In
Dry matter %^2		1	3867327	0.0750			In
Tip speed (m/sec)^2		1	1	0.4804			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	5215379	0.0433			In
Tip speed (m/sec)		1	1	0.3864			In
Flow rate (kg/h)		1			0.2843	0.6055	Out
Dry matter %		1			0.2785	0.6092	Out
Dry matter %*Tip speed (m/sec)		1			0.0151	0.9047	Out
Flow rate (kg/h)^2		1			0.0049	0.9458	Out
Dry matter %*Flow rate (kg/h)	Step Number 6	1	5779791	0.0333			In
Dry matter %^2		1	4022710	0.0680			In
Tip speed (m/sec)		1	3671470	0.0795			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	5390150	0.0387			In
Tip speed (m/sec)^2		1			0.5335	0.4804	Out
Flow rate (kg/h)		1			0.2797	0.6074	Out
Dry matter %		1			0.1340	0.7213	Out
Dry matter %*Tip speed (m/sec)		1			0.0001	0.9934	Out
Flow rate (kg/h)^2		1			0.0041	0.9499	Out
Dry matter %*Flow rate (kg/h)	Step Number7	1	1751405	0.2085			In
Dry matter %^2		1	0.6016	0.4519			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1	1425815	0.2538			In
Tip speed (m/sec)		1			3671470.0000	0.0795	Out
Tip speed (m/sec)^2		1			3300169.0000	0.0943	Out
Flow rate (kg/h)		1			3453042.0000	0.0878	Out
Dry matter %		1			0.2508	0.6256	Out
Dry matter %*Tip speed (m/sec)		1			3196919.0000	0.0990	Out
Flow rate (kg/h)^2		1			0.6262	0.4441	Out
Dry matter %*Flow rate (kg/h)	Step Number 8	1	1497274	0.2413			In
Flow rate (kg/h)*Tip speed (m/sec)		1	0.9079	0.3568			Removed
Dry matter %^2		1			1	0.4519	Out
Tip speed (m/sec)		1			0.3034	0.5911	Out
Tip speed (m/sec)^2		1			0.2581	0.6200	Out
Flow rate (kg/h)		1			0.1432	0.7112	Out
Dry matter %		1			0.4754	0.5026	Out
Dry matter %*Tip speed (m/sec)		1			0.0218	0.8850	Out
Flow rate (kg/h)^2		1			0.0007	0.9791	Out
Dry matter %*Flow rate (kg/h)	Step Number 9	1	1	0.4151			Removed
Flow rate (kg/h)*Tip speed (m/sec)		1			0.9079	0.3568	Out
Dry matter %^2		1			0	0.8162	Out
Tip speed (m/sec)		1			0.0003	0.9866	Out
Tip speed (m/sec)^2		1			0.0003	0.9868	Out
Flow rate (kg/h)		1			1017731.0000	0.3302	Out
Dry matter %		1			0.0790	0.7828	Out
Dry matter %*Tip speed (m/sec)		1			0.0329	0.8587	Out
Flow rate (kg/h)^2		1			0.5343	0.4769	Out
Dry matter %*Flow rate (kg/h)	Step Number 10	1			1	0.4151	Out
Flow rate (kg/h)*Tip speed (m/sec)		1			0.1052	0.7501	Out
Dry matter %^2		1			0	0.5693	Out
Tip speed (m/sec)		1			0.0015	0.9696	Out
Tip speed (m/sec)^2		1			0.0000	0.9967	Out
Flow rate (kg/h)		1			0.1354	0.7180	Out
Dry matter %		1			0.3880	0.5427	Out
Dry matter %*Tip speed (m/sec)		1			0.2372	0.6333	Out
Flow rate (kg/h)^2		1			0.1887	0.6702	Out