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# Deliverable report for

# GAIN

**Green Aquaculture Intensification**

Grant Agreement Number 773330

# Deliverable D1.6
# Title: Algae strains as multifunctional fish feed ingredients

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**WP 1 – Production and Environment**
**Task 1.1 – Algae as multifunctional feed components**

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# GLOSSARY OF ACRONYMS

| Acronym | Definition |
|---|---|
| $CO_2$ | Carbon dioxide |
| DW | Dry Weight |
| EPA | Ecosapentanoic acid |
| DHA | Docosahexaenoic acid |
| g/l | Gram per litre |
| ICP-OES | Inductively coupled plasma atomic emission spectroscopy |
| OD | Optical density |
| PT | *Phaeodactylum tricornutum* |
| QY | Quantum yield |
| Se | Selenium |
| Zn | Zinc |
| LC-PUFAs | Long-chain polyunsaturated fatty acids |



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# Executive summary

This deliverable reports the results from activities carried between M13-M20 of Task 1.1, WP1. With the objective of increasing Zinc (Zn) and Selenium (Se) content in microalgal biomass, first *Nannochloropsis* sp. was selected as model strain to study Zn and Se uptake. Growth conditions using different medium compositions were tested and successfully optimized, in order to increase the inclusion level of both Zn and Se into microalgal biomass.
Overall results showed that Se is actively uptaken by microalgae, leading to reach a Se concentration of 100 mg/kg of biomass; this value is in agreement with the target values set for the GAIN project (Appendix 1). Moreover, high concentrations of Se present in the growth medium (> 5.92 mg/L) lead to growth inhibition, showing toxicity of this mineral for microalgal cells. Zn is also uptaken by microalgal cells, and attempts to increase Zinc accumulation concentration led to reach Zn concentrations up to 84 mg/Kg of biomass; however uptake levels are less subjected to the increasing Zn concentration in the medium, suggesting that homeostasis mechanisms may prevent Zn iperaccumulation and toxicity. The values of Zn incorporated into microalgal biomass resulted to be competitive when compared to other microalgal species analysed. The medium composition containing the proper Zn and Se concentration, was determined and the growth condition established, thus *Nannochloropsis* sp was upscaled in our pilot facility AlgaePARC. Upscale of cultivation is currently done, in order to provide biomass to conduct Se and Zn inclusion and fish feed trials, in connection with GAIN partners and other WPs. Full details coming from this deliverable will be reported in forthcoming papers in scientific journals using OpenAcess to ensure optimal dissemination of the results.

# 1. Introduction

## 1.1 Background information

**GAIN project**

Green Aquaculture Intensification in Europe (GAIN) (www.unive.it/gainh2020_eu) is a project financed by the European Union under the Horizon2020 framework. The project is run by a consortium of 20 partners from a variety of professional backgrounds and spanning across 11 different countries, including Canada and China. The Consortium expertise are complemented by that of a US International partner. The composition of GAIN Consortium and the project structure are given in Annex 1; in Annex 1 is present also the targets for WP1, task 1.1. GAIN's primary aim is to assist the ecological intensification of aquaculture in the European Union (EU) and the European Economic Area (EEA), with the dual objectives of increasing production and competitiveness of the industry, while ensuring sustainability and compliance with EU regulations on food safety and environment.



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**Intensification of aquaculture through sustainable fish ingredients**

The global demand for aquaculture is increasing, in 2014 farmed fish surpassed wild catch (Golden *et al.*, 2016). In 2015, the EU already covered 3% of the total world production of which 80% still came from wild products (European Commission, 2017). Currently, EU-production still accounts for less than 50% of its total demand, meaning that there is still an opportunity to increase the EU self-sufficiency through domestic production (European Commission, 2017). The intensification of aquaculture should be achieved in a sustainable way which ensures not only an increase in economic growth but also environmental sustainability (AF*D et al.*, 2017). Therefore, several management approaches have been developed to deal with risks associated to aquaculture development (AFD *et al.*, 2017). Sustainability recommendations include sourcing fishmeal and fish oil from countries that implement responsible forage fishery (Simard and le Gouvello, 2017). A sustainable intensification of aquaculture in Europe is thus, underway.

**Fish formulation and minerals in feed ingredients**

Fish formulation is very important to ensure a sustainable fish production process. Fish require both essential nutrients and energy, thus their diet must be balanced in terms of proteins, carbohydrates, fatty acids, pigments, vitamins, and minerals (Lall and Tibbetts, 2009), (Lucas and Southgate, 2012). The majority of the ingredients utilized in feed formulation comes from three major group: Fish meal, Animal meal, such as Processed Animal Proteins (PAPs), and Plant meal made of plant-based oils, cereal (Figure 1). However, not all nutrients are digestible and there are nutrient losses through gill and urine excretions as well as in the faeces (Lucas and Southgate, 2012). Furthermore, an excess or lack of certain minerals as well as proteins will affect the fish skin and the composition of its body fluids and tissues (Lall and Tibbetts, 2009). Fish meal is the preferred ingredient in aquafeed because it contains the optimal amino acid and lipid content for healthy fish (in terms of amino acids which cannot be synthesised and long-chain polyunsaturated fatty acid, such as omega 3). However, ensuring that fishmeal sources are sustainable regarding over-exploitation of specific wild-fisheries is a challenge (SPAROS Lda., 2014), (Naylo*r et al.*, 2000). Impact on wild-fisheries is not just measured by the actual caught fish but the habitat and biodiversity of species, which have a major ecological impact (Naylo*r et al.*, 2000). Moreover, there has been a concern that some wild fishes have high levels of heavy metals such as mercury, dioxins and Polychlorinated biphenyl (PCBs) (Naylor *et al.*, 2009).

Once looking into using animal or plant-based proteins, there is a drive towards certain nutritional characteristics such as low levels of starch, fibre and ash (Naylor *et al.*, 2009). Animal base material also lack in supplying enough amount of micronutrient such as trace minerals, thus these needs to be introduced by inorganic mineral mixes (SPAROS Lda., 2014). Land-crop materials have been seen as sustainable alternatives to marine proteins and lipids used in aquafeed (Simard and le Gouvello, 2017). The main plant materials include barley, wheat, canola, soybean (SPAROS Lda., 2014) and (Naylor *et al.*, 2009), however, most plants have less sulphur-containing amino acids (cysteine and methionine), which can lead also to dietary imbalances. Furthermore, there is a concern regarding the palatability of the feed once adding plant feedstuffs to the feed and the increase of fish excretion and waste (Naylor *et al.*, 2009).

Once providing these ingredient sources to the fish through fish formulation, a special care



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must be taken into supplementing the diet with the necessary trace minerals and increasing the nutrient utilisation (Naylor et al., 2000 and 2009). Once fish feed is supplemented with certain trace minerals, the fish immune system is stronger, and they are able to heal much faster. Most of the time, industrial trace element pre-mixes are added during fish feed preparation, which contain inorganic forms that are not readably absorbed. These forms include Zn oxide, Zn sulphate and sodium selenite (Sparos ltd 2014, Rider et al., 2010, Dean et al., 2007). Concerns about the impact of copper, zinc and cadmium on benthic communities led to the implementation of regulatory/maximum levels of trace mineral concentrations in fish feed formulation (Rider et al., 2010), which however, are lower than fish dietary requirements. Therefore, there has been scientific and industrial interest in looking for better ways to add certain micronutrients into the feed and increase their bioavailability (Rider et al., 2010). For example, Se bioavailability is higher for Fish feed containing Se enriched-yeast, compared with feed including sodium selenite (Rider et al., 2010). Another study by Wang showed that antibody production increased as the amount of Se intake increased (Wang et al, 1997). The search for more bioavailable organic forms in still ongoing.

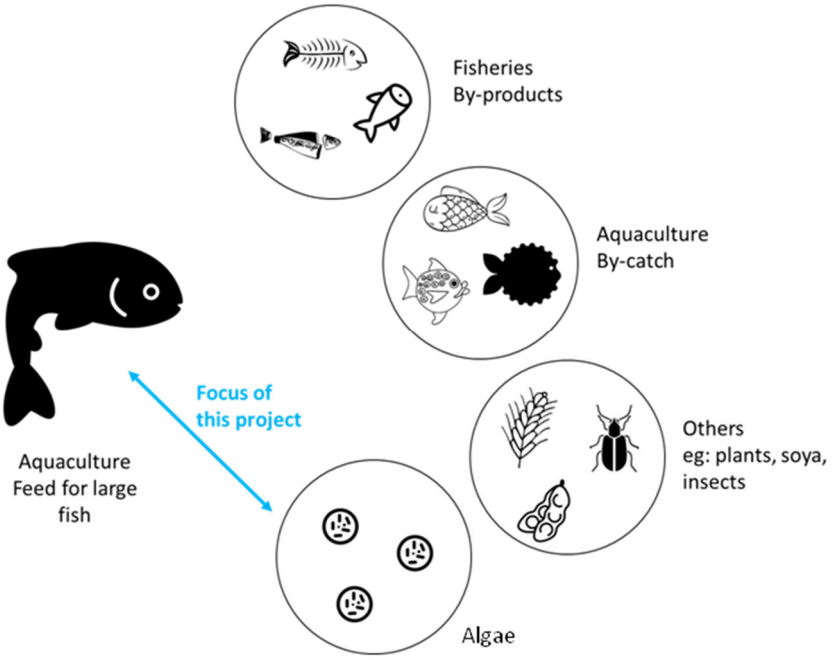


*Figure 1 – Ingredients used in fishfeed and the use of algae in this project as a microingredient.*

High-quality feed is expensive but also necessary to ensure a healthy diet, therefore sources such algae are seen with a particular interest as they can also be used as feed ingredient supplying Zn and Se minerals (Craig and Helfrich, 2002), (SPAROS Lda., 2014). Microalgae play a fundamental role as the base of the marine trophic chain and are involved directly or indirectly in the development of shellfish, molluscs and finfish (Becker, 2013) Shields and Lupatsch, 2012). These phototrophic microorganisms could be a great candidate to help solving challenges related to micro-nutrient requirements in aquaculture. They naturally contain a variety of compounds of interest such as proteins, lipids, sugars, carotenoids and minerals (Borowitzka, 1997) Gouveia et al. 2009, Shah *et al.*, 2018). Seaweeds (macroalgae) can also be used as feed ingredient supplying Zn and Se minerals; in addition, they could



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provide binder effects due to sulphated polysaccharides and stimulate fish immune system via beta glucans.

## 1.2 Task aim

Task 1.1 focuses on the selection of algae strains suitable as multi-valuable fish feed (Figure 1). In particular, it is investigated the use of algae as feed ingredients, in order to increase the bioavailability of Se and Zn, by incorporating these two minerals in the algae cells as organic mineral compounds (Figure 2).

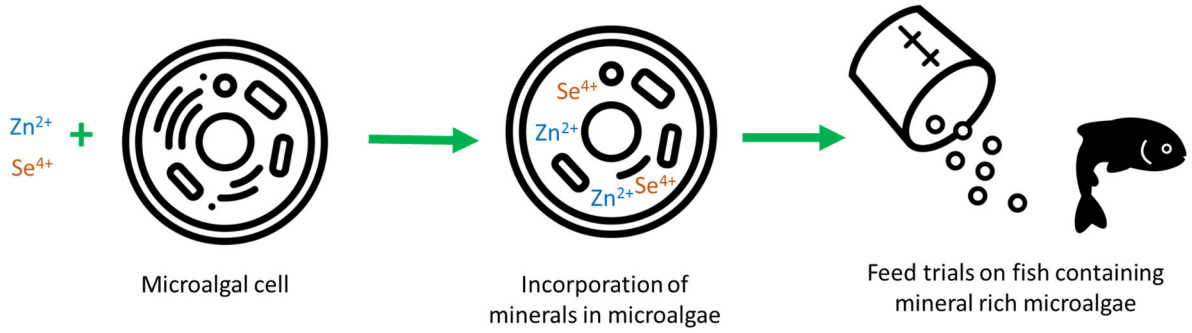


*Figure 2 – Outline of mineral incorporation in microalgal biomass, fish feed and used for fish trials.*

A better Zn and Se incorporation in the fish flesh will result in less waste of these 2 minerals in the wastewaters and sludge coming from aquaculture. As regards zinc, with GAIN partners we aim to reduce its typical concentration in the sludge from Atlantic salmon land-based farms from 300-600 mg $kg^{-1}$ to below 150 mg $kg^{-1}$, by improving Zn incorporation in the fish. If this target would be achieved, the sludge could be processed and turn into a valuable fertiliser. In parallel, we aim to demonstrate an improved uptake and retention efficiencies of zinc and selenium when incorporated in the fish feed as organically-bound to algal biomass. The increase in the amount of Zn and Se strengthens the immune system and reduces the likelihood of disease, which may require the use of pharmaceuticals.

An enhanced assimilation of Zn and Se in the fish will therefore 1) promote fish health; 2) comply with EU regulatory requirements (implementing regulation (EU) 2016/1095 and EC 1831/2003 and amendments) that limit the amount of inorganic Zn and Se in feeds of 180 mg/kg and 0.5 mg/kg (Khan et al, 2017). 3) Reduce the level of waste of these two minerals in the sludges.

## 1.3 Strain selection for mineral optimization

For the selection of microalgal strains for Task 1.1., a literature mining with the constraints used was conducted and presented on Deliverable 1.2 (M0-M12). The full the selection criteria list and the reason behind can be found in Annex 2. 4 most suitable microalgal candidates were then selected for preliminary metal (Zn, Se) tolerance screening, and to be used for fish-feed incorporation. In Annex 2 is also present a table and a resume with the properties for the most suitable microalgal strains identified (Table A2.2. ).
A preliminary trial was done to assess the robustness the shortlist of these 4 short-listed



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microalgal species, in order to determine which strain was more resistant to changes in media composition, as high concentrations of nutrients can become toxic. This work was reported in the deliverable file D1.2 of the GAIN project. Briefly, strains were grown at different medium composition, which included different ratios and increased concentration of nutrients N, P, and mineral traces containing Zn, up to 20X, compared to the original recipe. Secondly, Se tolerance was assessed, as Se it is usually not present in the culturing medium, and growth rates and biomass production were assessed.
After these screening results, *Nannochloropsis sp.* was selected as model strain to further study and optimize Se and Zn accumulation, because it showed no visible growth inhibition at any of the concentration tested for Se, Zn and other nutrients, and it had the highest biomass accumulation. This deliverable reports the results obtained for *Nannochlorospis* mineral optimization in the following months M13-M20 in Task 1.1.

# 2. Methods

## 2.1 Culturing conditions

**Media**

The medium formulation was carefully adjusted to avoid precipitation issues at high mineral concentrations. The ionic species containing sulphur in the original formulation (Guillard, 1975) were substituted by chloride forms in order to easily manipulate sulphur concentrations by means of a single media ingredient ($Na_2SO_4$, final concentration 0.92 g/L). This medium is made from artificial seawater and was named chloride medium. Se is not part of most algal cultivation media, so additional trials on Se species and concentrations were performed first separately.

**Microalgal strains and culturing conditions**

*Nannochloropsis sp.* was ordered from culture collections and grown in the laboratory at the Bioprocess Engineering group at Wageningen University and Research. These marine strains were all cultured in the same media under standard light and $CO_2$ conditions. Cell were grown, if otherwise stated, in triplicate in 250 mL Erlenmeyer flasks with 150 mL culture for first screening and optimization experiments.

## 2.2 ICP-OES method development for microalgal biomass

The mineral analysis is an essential part of this project as it is at the core of the research itself. For this part of the project a new protocol for mineral extraction and detection needs to be optimized for the microalgal biomass analysis (Figure 3). The equipment used is an Inductively coupled plasma atomic emission spectroscopy (ICP-OES), available at Wageningen University. For the mineral analysis a biomass concentration of at least 3 g/L per sample is required, thus we established that a culturing period of 10 days is enough to obtain cell samples for mineral analysis.
Cells were harvested by centrifugation, the cell pellets washed to remove any medium residual



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and freeze-dried.
The washing procedure was optimized by analysing mineral content inside the biomass and the mineral content in the wash solution at each washing steps (4 times). This allowed us to determine how many washing steps are required to clean-up the biomass from trace elements coming from the growth medium, prior mineral analysis. Method optimization will be part of a manuscript, in preparation.
After washing steps, the freeze-dried biomass was processed through a microwave-assisted acid digestion. Subsequently, the samples were loaded into an inductively coupled plasma optical emission spectroscopy (ICP-OES) machine (PerkinElmer Avio® 5000) to determine total mineral content of the mineral of interest (Zn, Se).

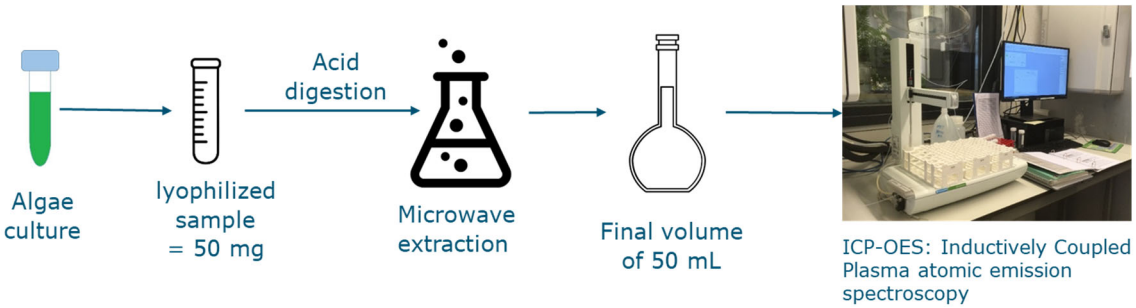


*Figure 3 - ICP-OES method development for lyophilised algal samples.*

**Ionic Species**
The main ionic species that will be studied through ICP-OES are Zn, Se and sulphur. Other minerals are also tested to assess any presence of cadmium or arsenic contamination when using lower grade chemicals at large scale cultivation. To translate the results obtained from the ICP into the correct values, calculations were made according to equation (1), for the biomass.

$$C_{AB} = C_m m_s \cdot D_d \cdot DICP \cdot 1000 \quad D_d = 1000 V_d \qquad (1)$$

$C_{AB}$ = Actual concentration in biomass sample, in mg [mineral] /g [biomass]
$C_m$ = Measured concentration, in mg [mineral] /L
$m_s$ = Mass of measured biomass sample, in mg [biomass]
$D_d$ = Dilution from digestion
D*ICP* = Dilution for ICP measurement
$V_d$ = Final digestion volume, in mL

## 2.3 Mineral uptake and optimization

**Zinc uptake**
Flasks were inoculated with *Nannochloropsis* sp. with a starting $OD_{750}$ of 0.5. for 12 days using the same basal media but different concentrations of zinc were used: 0.27, 6.86, 13.73, 27.46, 68.65 mg [Zn]/L. Experiments were performed in biological triplicates. Samples were collected daily to monitor: OD, Cell count and QY. After 12 days biomass samples collected and analysed for zinc content.



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**Testing Zn hindrance by buffer and chelating agents**

Experimental cultures were inoculated following the same process as the zinc uptake experiments. Basal media was prepared with and without the addition of buffer. Zinc stocks were prepared in two ways: using equimolar concentrations of zinc and EDTA and by preparing the zinc stocks in 0.1 M HCl. Samples were collected daily to monitor: OD, Cell count and QY. After 12 days biomass samples collected and analysed for zinc content.

**Growth rate and biomass productivity**

The growth rate and biomass productivity are calculated with equation (2) and (3). For the calculation of the growth rate, the variable time (t) is expressed in days (d), and dry weight measurements (g/L) are used as biomass concentration.

$$\mu = Ln(C_{end}) - Ln(C_0) t_{end} - t_0 \quad (2)$$

$$P = C_{end} - C_0 t_{end} - t_0 \quad (3)$$

$\mu$ =Growth rate ($d^{-1}$)
P = Production rate g[biomass]$*L^{-1}*d^{-1}$
$C_{end}$ =Concentration at $t_{end}$ g[biomass]$*L^{-1}$
$C_0$ =Concentration at $t_0$
g[biomass]$*L^{-1}$
$t_0$ =Start time (d)
$t_{end}$ =End time (d)

**Se uptake**

*Nannochloropsis* sp. cultures were inoculated following the same process as the zinc uptake experiments. Each selenium concentration tested consisted of :0 (control), 1.18, 2.37, 3.55, 4.73, 5.92, 7.1, 8.28, 11.83 mg/L. Samples were collected daily to monitor: OD, Cell count and QY. After 12 days biomass samples collected and analysed for selenium content.

## 2.4 Upscale of cultivation for GAIN fish feed trials

Scale up was performed in AlgaePARC by using in total 4 different cultivation steps: 500 mL Erlenmeyer's with 300 mL of culture, 25 L flat panel systems, 300 L indoor tubular reactors and the 1500 L LGem tubular system (Figure 4). Algal cultures were inoculated on average with a starting OD between 0.4 and 0.5 and cultivated over a period of 12 to 15 days up to a concentration above 2 g/L.



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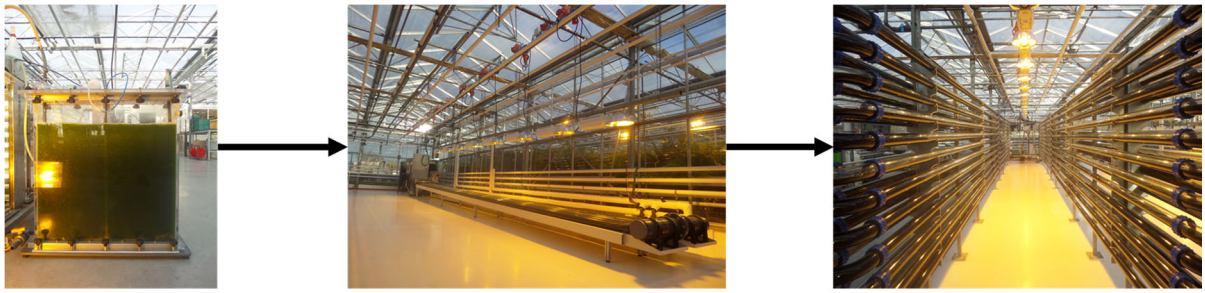


*Figure 2 – The scale up procedure of microalgal biomass for the fish feed trails on the different reactors available at AlgaePARC from the 25, to 300 to 1500 litre system. These systems will run during 2020 to achieve the necessary amount for the fishfeed trials.*

Samples were taken regularly to monitor the growth of the algae in the different systems. The harvested biomass was used to inoculate the following scale up system and both 1500 L and the 300 L were used for production and harvested by centrifugation (disk-stack EVODOS). The collected biomass was frozen, lyophilised and sent to the industrial partner for incorporation into fishfeed.

## *2.5. Statistical analysis*

Statistical analysis was done using SPSS (IBM SPSS Statistics 25). One-way analysis of variance (ANOVA) and post-hoc test (Tukey) were used with a 5% level of significance.



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# 3. Results and Discussion

## 3.1 Mineral uptake and optimization

**Zinc uptake**

A first trial was performed by growing *Nannochloropsis* sp. (n=3) at different concentrations of Zn in the medium, from standard concentration (0.27 mg/L), normally present in the growth medium as trace element, up to 4.12 mg/L (Deliverable 1.2).

In order to improve Zn incorporation, higher concentrations were explored from 0.27 to 68.65 mg of Zn in the media (Figure 5).

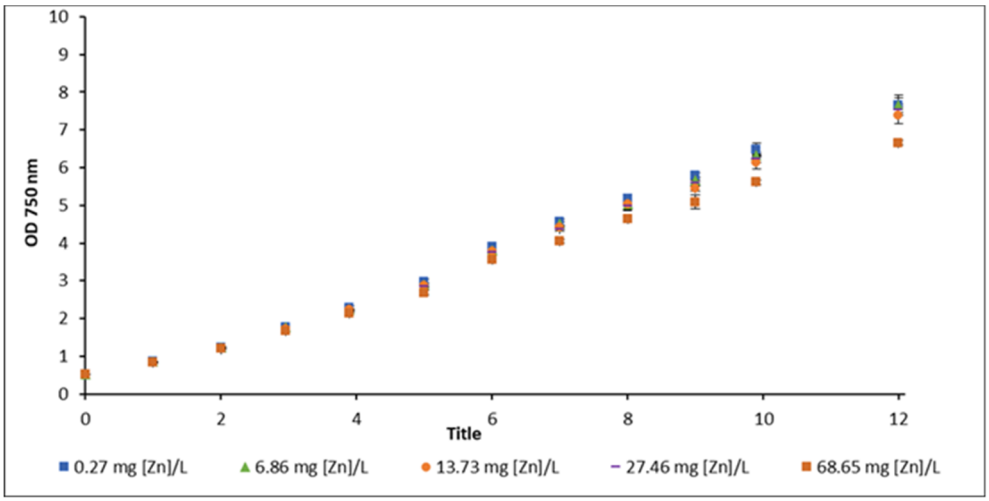


*Figure 5 - The OD of the algal cultures on the different Zn concentrations measured over 12 days. No visual effect is seen on growth from elevated Zn concentrations in the culture media.*

The maximum specific cell growth rate (during exponential phase) and the biomass productivity (during linear phase) were calculated for each concentration, these values can be found in Table 1.

These result show that there is no significant effect on maximum specific growth and biomass productivity for the Zinc concentrations of 0.27, 6.86, 13.73 and 27.46 mg [Zn]/L. However, the highest concentration of Zinc (68.65 mg [Zn]/L) significantly (p=0.026) hindered biomass growth.



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Table 1 - Maximum specific growth rate (during exponential phase) and productivity (during linear phase) for each different Zn concentration studied.

| medium concentration (mg [Zn]/L) | maximum specific growth rate (d-1) | productivity (mg [biomass]/d) |
|---|---|---|
| 0.27 | 0.33±0.00 | 0.21±0.01 |
| 6.86 | 0.32±0.01 | 0.22±0.01 |
| 13.73 | 0.32±0.01 | 0.22±0.01 |
| 27.46 | 0.32±0.01 | 0.22±0.00 |
| 68.65 | 0.31±0.01 | 0.18±0.03 |

Mineral analysis was performed and an overview of the low screening and high screening concentrations is presented below (Figure 6).

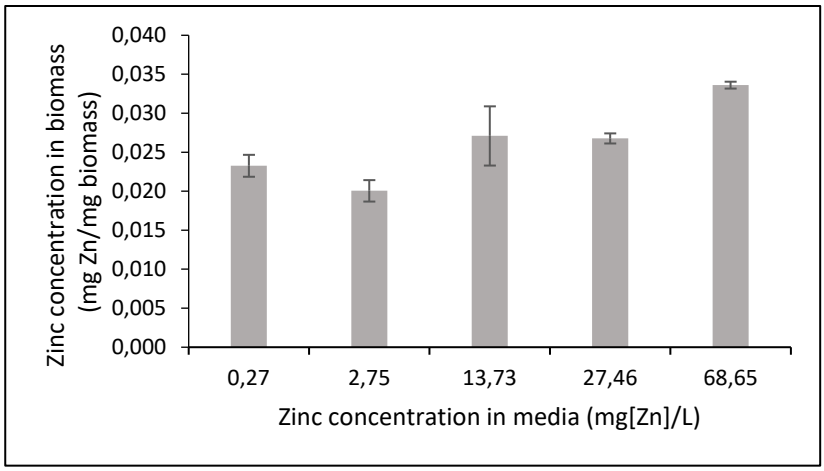


*Figure 6 - Zn concentration in the biomass in relation to the Zn concentration available in the media.*

Results showed there was no significant increase in Zinc uptake at most of the different Zn concentration tested (0.27-27.46 mg of Zn/L) and uptake improvement when microalgae were grown at 68.65 mg of Zn/L ($p < 0.001$) when compared to control (0.27 mg Zn/L).

**Testing Zn hindrance by buffer and chelating agents**

A multifactorial experiment was prepared using buffered cultures (with Tris) and unbuffered cultures and chelated media (Zn stocks prepared with and without EDTA addition).

The objective of this study was to understand if zinc availability was hindered by the use of buffers or chelating agents in the media. The hypothesis is that free ions of zinc ($Zn^{2+}$) may be not fully available in the medium, if chelated by other agents and not in the right oxidation state (Krezel and Maret, 2016). In this regard, buffers and chelating agents may be a factor hindering Zn availability.

Tris buffer is present in the medium formulation, therefore an optimization experiment was



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conducted by designing the medium to contain Tris (control) or no buffer at all (no Tris). The chelating agent EDTA is normally used in the Zn stocks, therefore a comparison was conducted between media formulated with Zn-EDTA in comparison to medium containing no EDTA ([1:0]). Chelated Zn stocks were prepared in equimolar concentrations with EDTA ([1:1]).

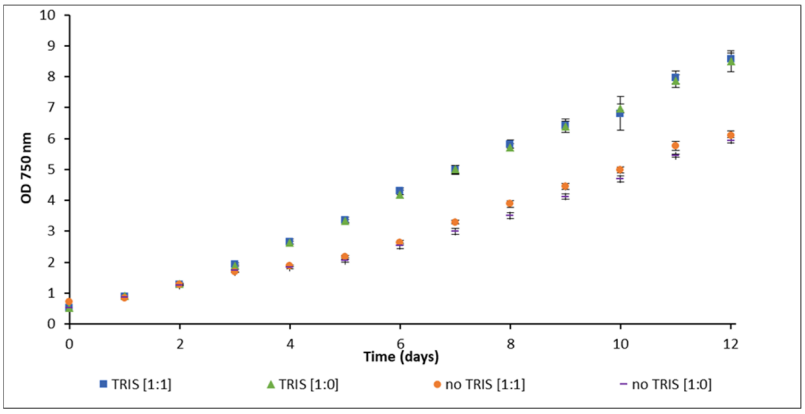


*Figure 7 - The OD of the algal cultures on buffered (Tris) and unbuffered media (no Tris) measured over 12 days. [1:1] represents media where Zn stocks were prepared in equimolar concentrations with EDTA and [1:0] represents media where Zn stocks had to EDTA addition.*

Specific growth rate and biomass productivity were also analysed for the cultures in the multifactorial experiment (Table 2). There is no measurable difference in growth between the cultures grown with the buffer Tris (with different molar fractions of Zn to EDTA) as can be seen in the growth curve in Figure 7 (Tris [1:1] and Tris [1:0]), indicating that the expected increase in free Zn ions did not negatively affect the *Nannochloropsis* sp. There was however a visible difference ($p<0.05$) between the cultures with and without buffer as can be seen in Figure 7 and Table 2. This shows a clear difference between growth with and without a buffer in the medium.

Table 1 - Maximum specific growth rate (during exponential phase) and productivity (during linear phase) for each treatment tested.

| **medium specification (mg [Zn]/L)** | **maximum specific growth rate (d-1)** | **productivity (mg [biomass]/d)** |
|---|---|---|
| Tris [1:1] | 0.36±0.01 | 0.25±0.01 |
| Tris [1:0] | 0.36±0.01 | 0.25±0.01 |
| No tris [1:1] | 0.35±0.01 | 0.18±0.01 |
| No tris [1:0] | 0.33±0.02 | 0.17±0.00 |



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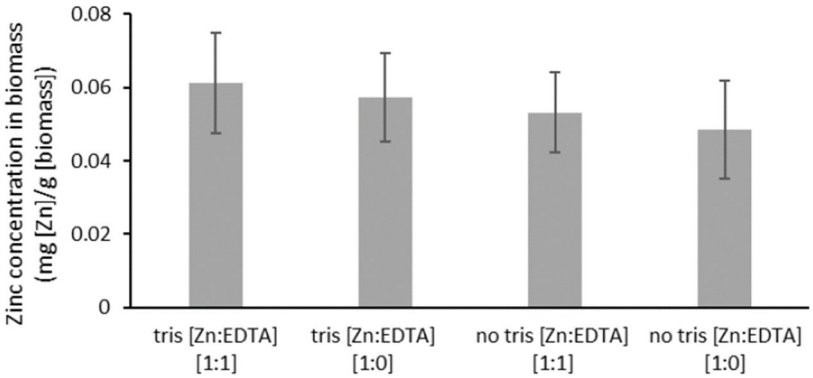


*Figure 8 - Mineral analysis of samples from each treatment tested, analysed at day 12 of growth.*

We performed mineral analysis per each treatment sample, harvested at day 12 of cultivation. As shown in Figure 8 there is no substantial difference in Zn accumulation among the conditions, therefore it was possible to rule out any negative interference effect due to the presence of buffering or chelating agents. Taken together, these results also suggest that buffered medium promote better growth compared to unbuffered medium, and chelating agents have no effect on Zn hindrance.

**Se uptake**

Se is not commonly used as trace element in microalgal growth medium, therefore it was important to assess working concentrations (Morlon et al. 2006, Zhao et al. 2019, Zheng et al. 2017, Zhong et al. 2017). The first screening was done in a range of concentrations from 0 (control) up to 3 mg/L. In this range, *Nannochloropsis* sp. does not show growth inhibition (Deliverable 1.2). Second set of experiments were performed with Se addition to the media ranging from 0 (control) to 8.28 mg/L (Figure 9). Cell hinderance was observed in concentrations higher than 4.73 mg/L. So, concentrations below that are considered most suitable for cultivation. This study allowed for an understanding of the working concentrations for Se in the media and samples were analysed to understand Se uptake in *Nannochloropsis* sp.

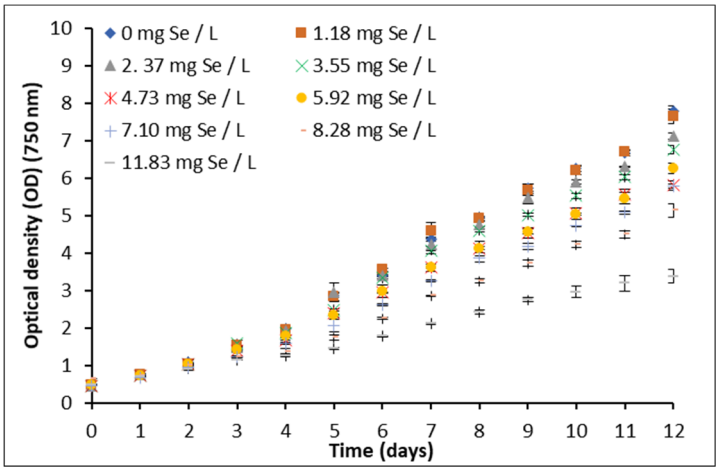


*Figure 9 – OD during cell cultivation under different Se concentration ranging from 0 to 8.28 mg Se/L for 12 days. Concentrations higher than 4.73 mg/L seems to hinger cell growth.*



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Mineral analysis was performed (Figure 10) and, unlikely for Zn, Se uptake increased as its concentration in the medium increased. Concentration of Se above 5.92 mg had impact on cell growth and biomass accumulation; because less biomass was produced, a noisy signal was observed for Se measurements on biomass treated with >7.1 mg/L of Se, as result of technical limitation of ICP-OES sensitivity.

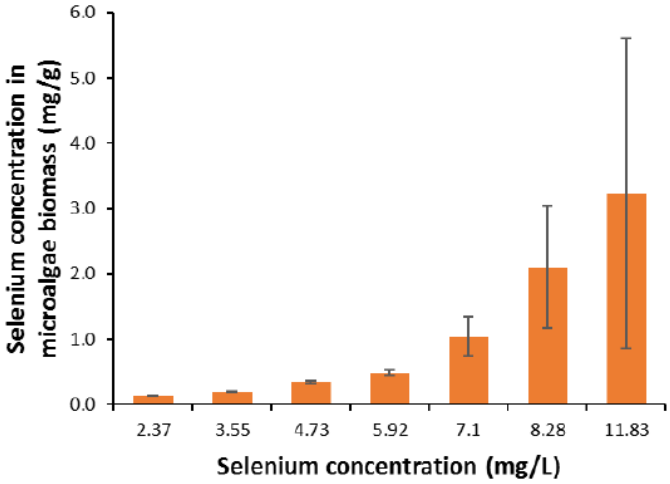


*Figure 3 – Mineral analysis of samples from each treatment tested, analysed at day 12 of growth. Digested biomass samples of the negative control (Se=0) have no Selenium and 1.18 mg Se/L cultures had selenium concentrations below detection limit, therefore these 2 points were not included in this graph.*

Sulfur content was also analysed by ICP-OES, to see the trend of accumulation of these 2 elements (Se and S) Figure 11. Selenium and sulfur are two closely related basic elements utilized in nature for a vast array of biochemical reactions. It is suggested that Se uptake may compete with sulfate salts ($Na_2SO_4$) present in the growth medium (Schiavon et al. 2017, Vriens et al., 2016). If true, this aspect could be used to further optimize medium composition to favour Se uptake.

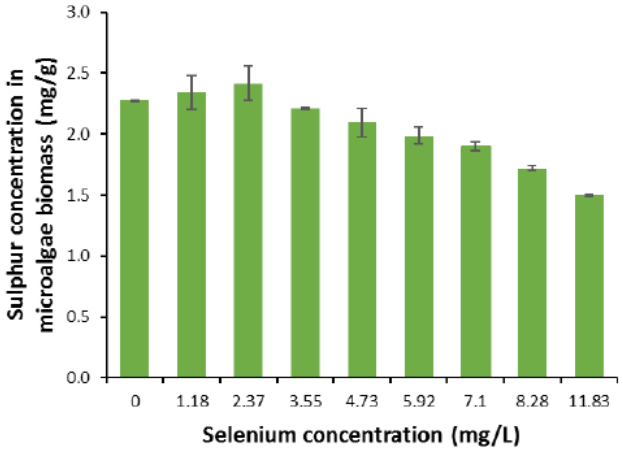


*Figure 4 – Sulfur analysis of samples from each treatment tested, analysed at day 12 of growth.*



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First analyses made showed that S presence in the biomass decreased when Se was introduced in the medium at 11.83 mg/L, compared to control conditions (0 Se present) and more experiments are needed to confirm this data. These results put the basis for further hypotheses on optimization and interaction among Se and S. In particular, a reduction of S presence in the medium may favour uptake of Se. However, S is considered an essential nutrient for microalgal growth, therefore S limitation is not ideal for growth and biomass accumulation (Sakarika et al. 2016). Therefore, future optimization experiments could focus on selective substitution of S content with Se content, to find the optimal S and Se concentrations that are not affecting the biomass productivity, while giving the highest amount of Se incorporated into the microalgal biomass.

**Selenium uptake**

After the initial screening, the experiments were conducted in a refined Se concentration range (0, 2.37, 3.15 mg/L) to define which concentration is the most appropriate for process scale-up (Figure 12).

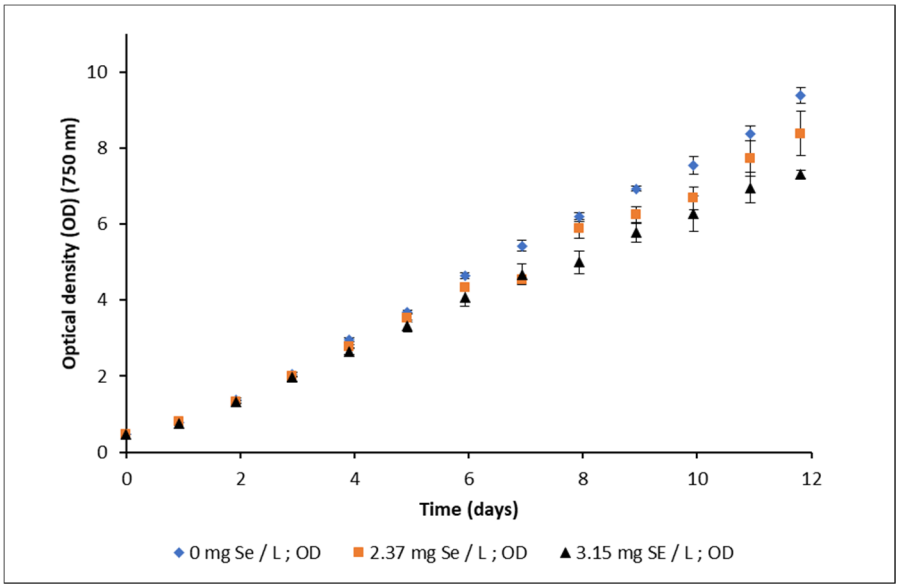


*Figure 12 – OD during cell cultivation under different Se concentration 0, 2.37, 3.15 mg/L.*

In these conditions, the addition of 2.37 mg/L and 3.15 mg/L of Se to the medium had a small effect on cell accumulation when compared to control cultures at 12 days, ($p=0.032$ and $p=0.001$, respectively). Moreover, the biomass samples analysed from this experiment resulted in Se accumulation of 0.131 ± 0.017 $g_{Se}/kg_{biomass}$ and 0.315 ±0.069 $g_{Se}/kg_{biomass}$, respectively. Thus, Se accumulation increased significantly ($p<0.05$) with the increase of Se in the media. Moreover, S content slightly increased with the selenium treatment. Control cultures had a sulphur content of 4.82 g Se/kg biomass. Cultures treated with 2.37 mg/L of Se



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had a S accumulation of 5.02 g Se/kg biomass which was higher than control (p=0.009). Finally cultures treated with 3.15 mg/L of Se had 5.14 g Se/kg biomass which was also higher (p<0.05) than the S accumulation in control cultures (Figure 13). However, these tested selenium concentrations are very similar and thus, could have a milder effect on sulphur uptake and accumulation in the cell. More experiments are needed to understand if there is a competitive uptake between these 2 chemical species.

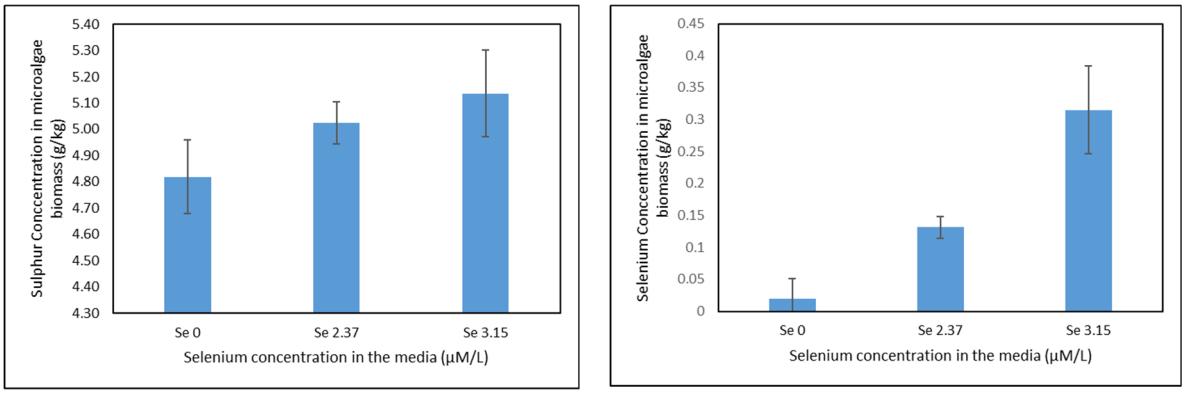


*Figure 13–Sulfur (S) and Selenium (Se) content in microalgal biomass grown at two different concentrations of Se. Panels from left to right: Sulfur determination for treatment at Se=0, Se=2.37 mg/L, Se=3.15 mg/L concentrations; Selenium determination for treatment at Se=0, Se=2.37 mg/L, Se=3.15 mg/L concentrations;*

Overall, it was possible to reach the target Se concentration in microalgae for the GAIN fish feed trials whilst ensuring a small effect on biomass accumulation (Annex1). Further experiments were set the biomass cultivation for fish trial purposes within the GAIN project, these will be presented in the form of a published open-access manuscript. Therefore, further experiments were set to understand the effect of two different Se species that can be used for upscaling and to confirm the working concentration of Se that can be used for upscaling the biomass cultivation for fish trial purposes within the GAIN project, these will be presented in the form of a published open-access manuscript. The media formulation was then made based on these experimental results and used for the upscale of microalgal growth for fish feed trials.

## 3.2 Upscale of cultivation for GAIN fish feed trials

*Nannochloropsis* sp. biomass cultivation was upscaled into AlgaePARC in order to produce 12 kg of selenium enriched dry biomass as part of the GAIN fish feed trials.

Large scale cultivation is a laborious endeavour and to date, the 1500 L system was harvested twice to provide enough biomass for the first feeding trials. In Figure 14 is a schematic representation of the different steps involved in the harvesting and preparation of the biomass. The algal biomass is firstly, dewatered by centrifugation, frozen and then lyophilised. Freeze-drying was preferred for this process in order to maintain the high nutritional composition of the algae and avoid any heat effect on sensitive cellular components such as fatty acids.



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*Figure 14– Representative scheme with pictures of the different steps involved in the harvesting and preparation of the biomass. A – large cultivation vessel, B – EVODOS centrifuge, C – Disk stack system of the EVOS centrifuge after being used with Nannochloropsis, D – collected biomass from the centrifuge, E – distribution of the algal biomass into smaller containers than will be frozen and later freeze-dried.*

Cultivation efforts will continue until mid 2020 and the results from this upscaling work will be used for the techno economic considerations, as part of the GAIN project.

## 3.3 Overview of Zinc and Selenium increase in *Nannochloropsis sp*.

In this study, we performed mineral uptake and optimization studies in order to evaluate and increase the Zn and Se content inside the biomass of *Nannochloropsis* sp. Little is known from literature about Zinc and Se accumulation in microalgae in general, being Se not usually present in the growth media of microalgae.

From the Se test, we observed that Se was actively incorporated into the microalgal biomass up at the different concentration tested: however, Se concentrations higher than 5.92 mg/L resulted deleterious for microalgal growth. The Se concentration obtained inside the microalgal biomass were in line with the GAIN target, for fish feed trial, i.e. 0.5 mg/kg of feed. A working concentration was selected and further studies were conducted to optimize upscale protocol. Therefore, the microalgal biomass produced will be used to substitute inorganic mix of Se in the fish feed trials carried pout during the GAIN project.

Zn uptake was limited, and we did not see any increase of Zn accumulation inside the microalgal biomass at increasing Zn concentration in the growth medium. A multiparameter experiment was performed in order to exclude the possibility that buffering and chelating agent present in the growth medium may hinder Zn availability: this showed no negative effect of these agents on Zn accumulation. Overall, the results suggest that uptake rate is still very low comparatively to the amount of Zn that is available, therefore homeostasis mechanism may take place. Future work should address this hypothesis and aim to understand what factor(s) may increase Zn uptake. In light of this result, the basal Zn uptake of other commercially available microalgal species, i.e. *Chlorella* sp. *Tetraselmis* sp., and *Spirulina* sp, was determined, in order to identify strains characterized by higher uptake were tested. (Figure 15, Table 3).



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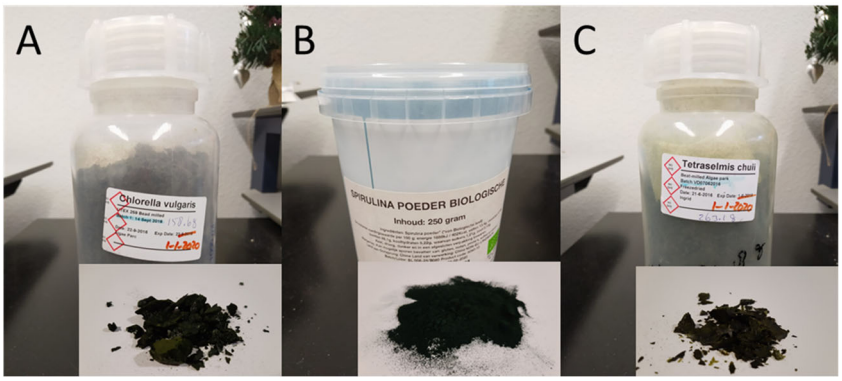


*Figure 15 – Chlorella sp. (A) and Tetraselmis sp. (C) were obtained from previously performed experiments at AlgaePARC and Spirulina sp. is an available product from a company (De Noten Shop, The Netherlands) (B).*

The mineral analysis showed that *Nannochloropsis* has basal higher zinc content (58.15 mg/kg), compared to the other microalgal biomass sources. *Nannochloropsis* Zn optimization efforts conducted in this study brought to 1.4 fold increase of Zn in *Nannochloropsis* biomass (up to 83.80 mg/kg). This value is still low for a full substitution of Zn supply in the fish feed (180 mg/ kg of feed) by using 0.5% microalgal biomass incorporated in the feed. However, algal content in the feed incorporation could be increase up to 30% (Sparos). Thus, with a higher incorporation of microalgae in the fish feed, an additional ~6X increase of Zn content would be sufficient to have full supply of Zn by microalgal biomass.

Table 3 - Mineral analysis performed on commercially available strains and the *Nannochloropsis* strain used in this project.

| Commercial strains | Mineral composition | |
|---|---|---|
| | mg **Zn**/kg biomass | mg **Se**/kg biomass |
| *Nannochloropsis* sp. * | 58.15 | - |
| *Nannochloropsis* sp. ** | **83.80** | **100.00** |
| *Chlorella* sp. | 29.74 | - |
| *Tetraselmis* sp. | 28.31 | - |
| *Spirulina* sp. | 13.38 | - |

* values obtained as a baseline in our studies
** values reached during experiments

A manuscript describing in more and full detail the work conducted is under preparation, because up-to date, very little information is available regarding mineral accumulation and uptake in salt water strains.

Carried out by our GAIN partner, SHP, Se incorporation was achieved for macroalgal biomass (deliverable D1.2) After selenization screening, it was found out that *Saccharina latissima* accumulated selenium accumulate 213.33+23.25 mg $kg^{-1}$, whereas *Laminaria digitata* and *L.*



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*hyperborea* exhibited a selenium content of 350+82.87 mg $kg^{-1}$. These macroalgae will be also used during the fish feed composition and trials (Annex 1).

## 3.4 Overview on algal biomass composition and features

The main focus of Task 1.1. is to screen and optimize algal strains with high Zn and Se content, in order to be used as multifunctional feed in aquaculture industry.
Overall, algae range from 20-60% on protein content % of dry weight, 10-50% on lipids, 15-30% on sugars, depending on the species and cultivation conditions (Borowitza et al. 1998, Cho et al. 2001, SPAROS, 2004, Han et al., 2014, Niccolai et al. 2019). These major component ranges are similar to fishmeal composition, therefore they naturally pose as a good alternative substitution for fish meal. Moreover, they contain pigments such as chlorophyll and other carotenoids of antioxidant properties (e.g. astaxanthin, fucoxanthin) and are a good source of vitamins and minerals (Cardozo et al 2007, Yaakob et al. 2014, Spalaorte et al. 2006, Niccolai et al. 2019, Muller Fuega et al. 2013). The presence of these molecules is considered a value added to the biomass. In the Annex 3 a full list of major components present in the algal biomass is reported. Table 4 resumes the major components in fish feed ingredients, compared with algae strains optimized *Nannochloropsis* biomass composition, including achievement made in this project.

Table 4 - Major components of feed ingredients and algae biomass. (Data taken from literature: Cho et al. 2001, SPAROS, 2004, Han et al., 2014, Niccolai et al. 2019, Murphy et al. 2013, Getachew et al. 2015, Ashour et al. 2019, market product values https://www.feedtables.com/content/fish-meal-protein-65, Poveda, Feed formulations 2014)

| Feed Source | Protein % dw | Lipid % dw | Sugar % dw | Other (ash, crude fibres) % dw | Mineral Zn mg/kg | Mineral Se mg/kg |
|---|---|---|---|---|---|---|
| Fishmeal | 50-60 | 2-20. | n.d. | 3-25. ash | 100-125. | 1 |
| Poultry meal | 66 | 10-15. | n.d | 10-15 ash | 60 | nd |
| Plant protein meal (wheat, corn, pea) | 60-80 | 1-6. | 6-16. | 0.5-4.5 ash, 0.5-1 crude fibres | 9-60. | 0-0.4. |
| Plant protein meal (soy, rapeseed, sunflower) | 20-40 | 2-4. | 1-6. | 6-7. ash, 3-26 crude fibres | 10-30. | 0.1-1. |
| **Algae (general)** | **20-60** | **10-50.** | **15-30** | **5 ash** | **20-100** | **nd** |
| Nannochloropsis spp. (*GAIN study) | 20-25 | 40-50 | 18-20 | 5 ash, 10 crude fibres | **80 *** | **100 *** |
| Laminaria spp. (*GAIN study) | 8-14. | 1.5 | 50-60. | 30 ash, including 10-30 crude fibres | 10-100 | **300*** |
| Saccharina ssp. (*GAIN study) | 8 | 1.3 | 60 | 20 | 3-5. | **200*** |



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From this table it is possible to consider that *Nannochloropsis* can be a perfect lipid source for fish feeds, having high content of PUFAs, such as EPA (Annex 2, Table A3.3), while macroalgae such as *Laminaria spp.* and *Saccharina spp.* being high in sugar content, can be incorporated in the fish feed aiming at providing good source of digestible sugars (Table 5).

# 4. Conclusions

We reported information about microalgae used as multifunctional feed, highlighting the best properties of 4 selected strains: *Tetraselmis, Dunaliella, Phaeodactylum* and *Nannochloropsis* sp (Annex 2-3). *Nannochloropsis* sp. was used as a model strain to study zinc and selenium uptake. The growth conditions and medium composition, were successfully optimized, in order to increase the inclusion level of both Zn and Se. A concentration of Se of 100 mg/kg of biomass was reached, which is in agreement with the target values set for the GAIN project. Zn concentration was also increased to values up to 84 mg/Kg of biomass: these values are competitive, compared to other microalgal species analysed, being up to 6 times higher than the Zn content present in commercially-available algal biomass (*Spirulina* sp.).
We selected *Nannochloropsis* sp for the upscale of the cultivation, in order to conduct Se and Zn inclusion and fish feed trials, in connection with GAIN partners and other WPs. Full details coming from this deliverable will be reported in forthcoming papers in scientific journals using OpenAcess to ensure optimal dissemination of the results.

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# ANNEX 1 - Target values for Zn and Se

Values from SPAROS (fishfeed company partner of GAIN Consortium) and European legislation were set for a maximum of 180 mg of Zinc per kg of feed and 0.5 mg of Se per kg of feed. Assuming an incorporation of microalgae of 0.5 % per total feed. One kg of feed contains 5 g algae. So:

Zn: .180 g Zn/5g algae = .036 g Zn/g algae
Se: .0005 g Se/ 5g algae = 0.0001 g Se/g algae

**Target GAIN Fish trial feed composition**

Feed formulation was made by SPAROS and will include the biomass from macroalgae (seaweeds) and microalgae, as described in the below table. Both biomasses have several benefits, and have been shown to increase food uptake (because more palatable for the fish) and fish faeces consistency (more solid, easy to treat for removal) -internal communication with SPAROS and SHP partners.

*Feed formulation for GAIN project*

| Ingredients needed | Macroalgae | Microalgae | |
|---|---|---|---|
| Trial | Kg | Kg | Feeds production |
| salmon | 30.4 | 7.6 | 2020 |
| seabream | 3.5 | 0.9 | 2020 |
| seabream | 3.3 | 0.8 | 2020 |
| trout | 5.9 | 1.5 | 2020 |
| turbot | 4.7 | 1.2 | 2020 |
| | 48 | 12.0 | |



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# ANNEX 2 – Literature mining for strain selection

**Selection criteria for literature mining**

To be used in aquaculture, microalgae species has to meet various criteria. It has to be easily cultured and nontoxic. It also needs to be of the correct size and shape to be ingested and to have a high nutritional qualities and a digestible cell wall to make nutrients available (Niccolai 2019, Spolaore, 2006). Several considerations were made as pre-selection criteria, based on literature mining, as presented in the scheme below.

Table A2.1 – Literature mining for algal strains suitable to start with mineral supplementation studies.

| Criteria | Explanation | Consideration |
|---|---|---|
| Robustness | Focus on microalgae that can be grown at large scale, are fast growers, and are less sensitive to outdoor fluctuating conditions (e.g. Temperature shifts), industrial relevance, and already usage in aquaculture | Economy and industrialization |
| Reported capability to accumulate metals | Strains used in bioremediation of heavy metals will be prioritized | Mineral optimization |
| Seawater vs freshwater microalgal strains | Marine microalgae were prioritized as the fish trials in GAIN project will focus on Atlantic salmon, seabream and seabass, as the most relevant species both in volume and value for the European and Mediterranean aquaculture; moreover, in a large scenario, marine microalgal biomass production will not compete with fresh-water and land usage, therefore it is more convenient from a socio-environmental perspective. | Socio-economy and industrialization |
| Fish digestibility | Strains without or with a weaker cell wall may be preferable for fish meal digestibility. | Fish feed improvement |
| Presence of other value-added molecules in the biomass | Priority on high content of omega 3 fatty acids, pigments/antioxidants, essential amino acids, storage compounds (i.e. sugars, triacylglycerides). The presence of these components will reduce the need for extra supplementation in fish feed. | Fish feed improvement |
| Cell size | Cell size is thought to have an impact in the cell capability to adsorb minerals, bigger cells have a larger contact surface to retain minerals. | Mineral uptake capability |



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Table A2.2- list of the most representative species of microalgae already used in aquaculture, Modified from Brown et al. 2002. (+ indicates level of popularity).

| | Bivalve molluscs | Crustacean larvae | Juvenile abalone | Zooplankton (used for crustacean, fish larvae) |
|---|---|---|---|---|
| Isochrysis sp. (T.ISO) | ++ | + | | ++ |
| Pavlova lutheri | ++ | + | | ++ |
| Chaetoceros calcitrans | ++ | ++ | | + |
| C. muelleri or C. gracilis | + | ++ | | + |
| Thalassiosira pseudonana | + | + | | |
| Skeletonema spp. | + | ++ | | |
| Tetraselmis suecica | + | + | | ++ |
| Rhodomonas spp. | + | | | |
| Pyramimonas spp. | + | | | |
| Navicula spp. | + | + | ++ | |
| Nitzschia spp. | | + | ++ | |
| Cocconeis spp. | | | + | |
| Amphora spp. | | | + | |
| Nannochloropsis spp. | | | | ++ |

Table A2.3. Microalgal Selection made in this study. A cross is representative that the microalgal strain has that characteristic.

| Algal Selection Criteria | Algal Strains | | | |
|---|---|---|---|---|
| | *Nannochloropsis oceanica* | *Dunaliella salina* | *Tetraselmis chuii* | *Phaeodactylum tricornutum* |
| Robust strain | X | X | X | X |
| Used in aquaculture | X | X | X | X |
| Easy to count cell number | X | X | X | |
| Rigid cell wall | X | | X | |
| Interesting components | Omega 3 (EPA) | Carotenoid rich | Starch | Omega 3 (EPA) |
| Cell size | 2-3 µm | 4–15 µm wide, 6–25 µm long | 9-10 µm wide 12-14 µm long | 2-3 µm wide, 15-19 µm long |

***Dunaliella spp.* properties**

**Biomass composition and relevance:** *Dunaliella spp.* are considered a rich source of natural b- carotene, which can accumulate this carotenoid up to 10% of the cellular dry weight under certain extreme environmental conditions, such as high light intensity, nutrient deprivation, high salinity, and extreme temperatures (Lamers et al. 2010, Borowitzka et al., 2007, Kleinegris et al., 2009, Wijffels et al. 2019). It is one of the few microalgae that does not contain a cell wall; this creates an advantage in terms of digestibility of the biomass, when used for feed purposes.

*Dunaliella* spp. are cultivated as feed ingredients for human consumption, and they are used for enhancing the colour of egg yolks (Ben-Amotzet al.1986; Moulton and Burford 1990) and the flesh and shell of aquatic animals to make them more attractive in the market (Pulz and Gross 2004). Although shrimps convert b-carotene to astaxanthin, therefore, astaxanthin can be used for the same more effectively (Boonyaratpalinet al.2001, Tafreshi et al. 2009). Moreover, it has been recently investigated for their essential amino acid profile values, which



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are close to human nutrition value reference (Hopkins et al. 2019, Del campo et al. 2007, Sui et al. 2019).
**Cultivation:** *Dunaliella* spp. can reach a biomass productivity of 0.3 g/L/d (Hopkins et al. 2019, Del campo et al. 2007, Sui et al. 2019, 2020). It can be produced in open-pond systems and found commercial space in Australia, China, Israel and India (200-400 ha) (Del campo et al. 2007). Usually, a two-phase growth is required to obtain high-content of b-carotene (>10% of dry weight. The two-phase growth means that cells are first grown under standard conditions until reaching a favourable biomass density, and then the culture is subjected to stress conditions, such as high salt, nutrient and light stress. These conditions are highly impacting the growth rate and biomass accumulation, therefore in the second phase biomass accumulation will not increase; this aspect needs to be taken into account for the economy and easiness of the cultivation systems.

***Tetraselmis spp.*** **properties**
**Biomass composition and relevance:** species of *Tetraselmis* are marine green microalgae, often reported high salt-tolerant (D'Adamo et al. 2014). This green microalga is motile, having 4 flagella, and it contains starch, considered digestible carbohydrate (10-20 %). This microalga contained also high amounts of protein (30-50%), but relatively low lipid content 7-10% (Pereira et al. 2019, Rahman et al. 2017). They are generally used in aquaculture in conjunction with diatoms as live feed, in order to ensure a suitable nutritional profile for the grazing animals, used then to feed fish, mollusc and crostacean larvae (Day et al. 1998, Concei et al. 2010). Copper tolerance was recently studied for this species (Kumar et al. 2017).
**Cultivation**: they can grow at cell densities >0.7 g/l and can be grown in different culturing format, at industrial scale, from photobioreactors to plastic bags, typically used in hatchery (Michels et al. 2014, Pereira et al. 2018, Ishika et al. 2019). However, due to the presence of 4 flagella, also *Tetraselmis* is shear-sensitive, therefore a care on the cultivation technique selection needs to be taken into consideration for upscaling.

***Phaeodactylum*** **spp. properties**
**Biomass composition and relevance:** *Phaeodactylum* is also considered a model diatom, and it is used extensively for study the physiology and metabolism of these microalgae (Jallet et al. 2016, Remmers et al. 2018). Its biomass composition contains 38 % of proteins, 11% of carbohydrates, most of which are considered not digestible sugars (fibers), 1-3% fucoxanthin pigment, and 20% lipids, under normal culturing conditions. *Phaeodactylum* is considered a good source of omega 3 fatty acid EPA and the antioxidant pigment fucoxhantin (Nicolai et al. 2019). As for *Tetraselmis, Phaeodactylum* is also used in aquaculture as live feed, in order to ensure a suitable nutritional profile for the grazing animals, to feed fish, mollusc and crostacean larvae (Marinho et al 2017). Although this diatom presents a silica-based cell wall, *Phaeodactylum tricornutum* was used in digestibility trial studies to replacing fish meal in adult mink (Skrede et al., 2011) and salmons (Sorensen et al. 2016), up to 6% of inclusion in the final feed, giving promising results in term of digestibility.
**Cultivation:** *Phaeodactylum* spp. are marine species and cultivated world-wide; they can be grown easily outdoor in closed reactors, reaching 0.3 g/l in density, however, its temperature sensitivity (optimal 18-22 C), require extra energy for cooling the systems (Kudo et al. 2000).



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***Nannochloropsis spp.* properties**

**Biomass composition and relevance:** *Nannochloropsis* spp. gained a lot of interest in the las few decades for studies of lipid production for biofuels and/or the production of long-chain polyunsaturated fatty acids, especially EPA (Borowitza et al. 2018). This microalgae is rich in lipids (40-60%), with high content of the omega 3 EPA (9-22%). Its biomass is produced and sold, with a niche of application for aquaculture, as green water treatment for fish and larval tanks, for rotifer diet. Its interest for aquaculture application is growing (Li et al. 2020), and the usage of this biomass for fish feed has been evaluated in recent studies in thilapia, shrimp, obtaining positive effect on growth performance and fatty acid profile (Lupatsch et al. 2018, Adissin et al. 2019).

**Cultivation:** *Nannochloropsis* is a robust strain for outdoor growth, can easily grow in different culturing format, especially in closed photobioreactors, and it was also also recently positively evaluated for outdoor growth in open ponds (Li et al. 2020).

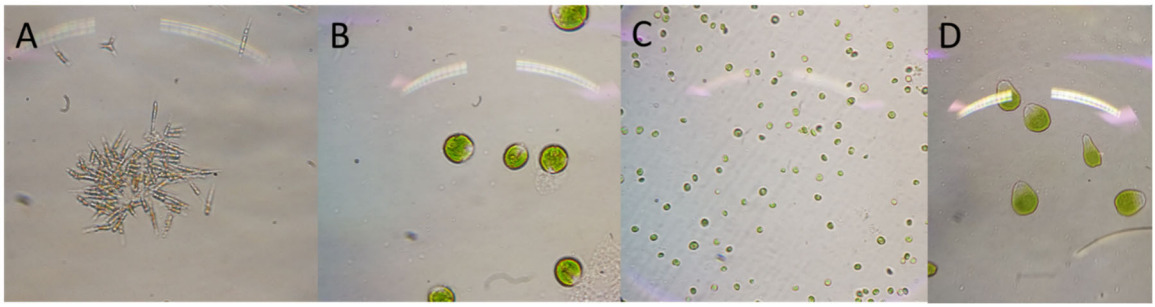


Figure A2.1 – Optical Microscopy images of the strains used in the project: *Phaeodactylum* sp. (A), *Tetraselmis* sp. (B), Nannochloropsis (C), *Dunaliella* sp. (D)



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# ANNEX 3 -Literature mining for microalgae as multi-functional feed ingredient

Microalgae are mainly eukaryotic photosynthetic microorganism belonging to different class and families, with over 30,000 species present. Microalgae can be divided in marine and freshwater species. Marine species naturally are found in oceans, seas, salty basins, while freshwater species populate lakes, and land-water sources (Heimann and Huerlimann, 2015). Microalgae are usually classified in three main groups: rhodophytes (red algae), chlorophytes (green algae) and phaeophyces (brown algae). Their biomass composition and nutritional values can vary quite extensively (Garcia et al. 2017), therefore a selection on the best microalgal species that can be considered as multifunctional feed is required.

## Starch and protein sources

The most studied microalgae belong to the group of Chlorophytes, which contain high level of proteins (from 40-58% on dry weight, depending on the strain) and accumulate starch as storage compound (10-30% on dry weight, depending on the strain and cultivation conditions) **Errore. L'origine riferimento non è stata trovata.**. The freshwater microalgae *Chlamydomonas reinhardtii* has been employed as model organisms for physiological and biochemical studies, including photosynthesis, and to date is the most well studied microalgal species (Salome' et al. 2019); however, it has less relevance for industrial purposes, as a scale-up process of these microalgae for large cultivation has not yet been established (Zedler et al. 2016). To date, among the freshwater species, only *Chlorella* is considered an industrial microalga, and it found the most commercial space as food ingredient and supply for human consumption (Guccione et al. 2014). Along with *Chlorella*, the freshwater cyanobacterium *Arthrospira spp*. (previously known as *Spirulina spp*.) is industrially exploited for food supplies and human consumption. *Dunaliella* and *Tetraselmis* are the most studied and interesting representative of marine green algae. Both these microalgae can be cultivated in large scale systems, and they find commercial application in aquaculture and as food ingredients (Spalaorte et al. 2006, Lamers et al. 2010, Muller-Fuega et al. 2003, Zittelli et al. 2006).



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Table A3.1 - Example of major chemical composition of green microalgae. (Modified from Demibras 2011)

Chemical composition of algae on a dry matter basis (%).

| Species of sample | Proteins | Carbohydrates | Lipids | Nucleic acid |
|---|---|---|---|---|
| *Scenedesmus obliquus* | 50–56 | 10–17 | 12–14 | 3–6 |
| *Scenedesmus quadricauda* | 47 | – | 1.9 | – |
| *Scenedesmus dimorphus* | 8–18 | 21–52 | 16–40 | – |
| *Chlamydomonas rheinhardii* | 48 | 17 | 21 | – |
| *Chlorella vulgaris* | 51–58 | 12–17 | 14–22 | 4–5 |
| *Chlorella pyrenoidosa* | 57 | 26 | 2 | – |
| *Spirogyra* sp. | 6–20 | 33–64 | 11–21 | – |
| *Dunaliella bioculata* | 49 | 4 | 8 | – |
| *Dunaliella salina* | 57 | 32 | 6 | – |
| *Euglena gracilis* | 39–61 | 14–18 | 14–20 | – |
| *Prymnesium parvum* | 28–45 | 25–33 | 22–38 | 1–2 |
| *Tetraselmis maculata* | 52 | 15 | 3 | – |
| *Porphyridium cruentum* | 28–39 | 40–57 | 9–14 | – |
| *Spirulina platensis* | 46–63 | 8–14 | 4–9 | 2–5 |
| *Spirulina maxima* | 60–71 | 13–16 | 6–7 | 3–4.5 |
| *Synechoccus* sp. | 63 | 15 | 11 | 5 |
| *Anabaena cylindrica* | 43–56 | 25–30 | 4–7 | – |

## Pigments content on microalgae

Microalgae naturally contain several pigment and carotenoids, which are seen as value-added molecule to the biomass composition. Indeed, the global carotenoids market is projected to increase because of the rising consumer awareness about health benefits offered by various carotenoids, and the shift towards healthy and natural food products (Global Industry Analysts, Inc http://www. strategyr.com/Carotenoids_Market_Report.asp/; Yaakob et al. 2014). Chlorophylls are pigments present in all microalgae. Other valuable pigments are astaxanthin and fucoxanthin, with their antioxidant properties, luthein, phycobilliproteins and b-carotene (Naguib, 2000, Cuellar-Bermudez et al. 2015), Table A3.2.

Lutein is present in green microalgae especially *Chlorella sp., Scenedesmus sp. and Muriellopsis sp*. This yellow carotenoid is used for the pigmentation of animal tissues and products and for the natural coloration of foods, drugs and cosmetics. Less studied for aquaculture purpose, this carotenoid has reported active role in stimulating the immune response and in delaying the progression of several chronic diseases, blindness and cataracts, and early atherosclerosis, in human studies (Landrum et al. 2001).

Astaxanthin and fucoxanthin are carotenoid of reported antioxidant activity. Astaxanthin can be use as pigmentation source in aquaculture, specifically to culture salmon, shrimp, ornamental fish and sea bream. Natural astaxanthin can be produced by *Haematococcus* microalgae, *Chlorella zofingiensis*, *Chlorococcum sp*. Fucoxanthin is a pigment responsible for photosynthesis in brown microalgae and of great interest for the aquaculture and the pharmaceutical industries; for aquaculture in interesting as part of fish and larvae diets, having bioactive properties, such as anti-oxidant, anti-cancer and anti-obesity element (Mikami & Hosokawa, 2013a; Muller-Feuga et al., 2007).

Phycobilliproteins are found especially in cyanobacteria such as *Arthrospira* (i.e *Spirulina*) and red algae (i.e. *Porphyridium*) and can be divided into phycocyanin (blue pigment), phycoerythrins (red pigment), and allophycocyanin (pale-blue pigment). *Spirulina* sp. is a



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source of c-phycocyanins and allophycocyanins; this cyanobacterium is already used as food and feed ingredients and contains 55%-70% protein, 6%-9% fat, and 15%-20% carbohydrate, and pigments (Boussiba and Richmond, 1979).

Table A3.2 - Values for total carotenoid and specific carotenoid or pigment present in microalgae. (Modified from Ambati et al. 2018.)

| Algal species | [#]Pigment (%) | [#]Major pigment or total carotenoids | References |
|---|---|---|---|
| *Chlorella zofingiensis* | 0.7% | AX | Bar et al., 1995 |
| *Coelastrella striolata Var. multistriata* | 0.15% | AX | Abe et al., 2007 |
| *Dunaliella salina* | 3–13% | BC | El-Baz et al., 2002; |
| *Chlorella zofingiensis* | 0.9% | BC | Bar et al., 1995 |
| *Coelastrella striolata Var. multistriata* | 0.7% | BC | Abe et al., 2007 |
| *Spirulina platensis* | 70–80% TC | BC | Miranda et al., 1998; El-Baky et al., 2003; Jaime et al., 2005; Ranga Rao et al., 2010 |
| *Chlorella pyrenoidosa* | 0.2–0.4% | LT | Wu et al., 2007 |
| *Botryococcus braunii* | 0.16% | LT | Tonegawa et al., 1998 |
| *Botryococcus braunii* | 75% TC | LT | Ranga Rao et al., 2006; 2007a; 2010a; 2010b; 2013b |
| *Chlorella vulgaris* | 45% TC | LT CX | Mendes et al., 2003; Singh and Gu, 2010; Chacon-Lee et al., 2010; Cha et al., 2010 |
| *Phaeodactylum tricornutum* | 1.65% | FX | Ragni et al., 2007; Kim et al., 2012; Dambek et al., 2012 |
| *Isochrysis aff. galbana* | 1.8% | FX | Kim et al., 2012 |
| *Cylindrotheca closterium* | 0.5% | FX | Rijstenbil et al., 2003 |
| *Odontella aurita* | 2.2% | FX | Xia et al., 2013 |
| *Coelastrella striolata Var. multistriata* | 4.7% | CX | Abe et al., 2007 |
| *Chlorella zofingiensis* | 25% TC | CX | Bar et al., 1995 |
| *Chlorella vulgaris* | 36% TC | CX | Li et al., 2002; Singh et al., 2010; Chacon-Lee et al., 2010; Kong et al., 2012 |
| *Botryococcus braunii* | 0.17% | ECN | Tonegawa et al., 1998 |
| *Nannochloropsis* sps | 0.1% | TC | Lubian et al., 2000; Macias-Sanchez et al., 2005; Forjan et al., 2007; Nobre et al., 2013; Solovchenko et al., 2014 |
| *Scenedesmus* sps | 0.69% | TC | Qin et al., 2008; Ceron et al., 2008; Pirastru et al., 2012; Chan et al., 2013; Guedes et al., 2013; Ho et al., 2014; |
| *Chlorococcum* sps | 0.25% | TC | Zhang and Lee, 1997; Zhang et al., 997;Masojidek et al., 2000; Yuan et al., 2002; Sivathanu and Palaniswamy, 2012; |

[#]carotenoid contents in algae species varied upon their culture conditions; AX, astaxanthin; BC, β-carotene; LT, lutein; CX, canthaxanthin; ECN, echineone; TC, total carotenoids.

## Lipid and long chain poly-unsaturated fatty acids content

The primary lipid source for aquaculture feeds is traditionally supplied by marine oils content in fishmeal production. This meal contains long-chain polyunsaturated fatty acids (LC-PUFAs), such as omega 3 Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), which are reported to be accumulated in the fishes, having been unequivocally associated with a protective role against a number of human diseases (SPAROS, Ltd. 2014, Alhazzaa et al. 2019, Ahmmed et al. 2019). Because plant- and animal-based oils do not contain EPA or DHA, the inclusion of these lipid sources in fish feed causes a reduction in EPA and DHA contents. Fish oils such as from salmon tuna, krill and copepod oil are available, and the range of LC-PUFAs in fish oils are from 8- 20% of total fatty acids. However, their use in aquaculture feeds is still limited by cost constraints. Moreover, their limited supply instigated the need to find and use other sustainable oil sources. Microalgae can be a game changer in the supply of lipids for fish feed, as they are natural sources of triglycerides and long chain poly-unsaturated fatty acids (e.g. omega 3 and omega 6) **Errore. L'origine riferimento non è stata trovata.**. Green microalgae either do not contain or they contain only trace amounts of LC-PUFAs, while brown microalgae such as *Nannochloropsis* sp. and the diatoms such as *Phaeodactylum sp., Thalasiossira spp.* are incredibly rich, and they have similar content compared to fish oils, specifically for EPA values (Table A3.3) (Khozin-Goldberg et al. 2011, Cuellar-Bermudex et al. 2015, Moomaw et al 2017).



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Table A3.3 - Total lipid content (% of dry weight) and EPA/DHA content (% of total fatty acid methyl esters) of diverse photoautotrophic microalgae belonging to different classes (Modified from Ryckebosch et al. 2012).

| Phylum | Species | Total lipids | EPA | DHA |
|---|---|---|---|---|
| Bacillariophyta | *Chaetoceros* | 9 – 40% | 8 – 22% | 0.03 – 5% |
| | *Phaeodactylum* | 15 – 40% | 2 – 36% | 0 – 3% |
| | *Skeletonema* | 3 – 25% | 13 – 36% | 1 – 6% |
| | *Thalassiosira* | 9 – 26% | 11 – 17% | 1 – 5% |
| Chlorophyta | *Tetraselmis* | 6 – 26% | 4 – 11% | 0 – 0.1% |
| Cryptophyta | *Cryptomonas* | 12 – 22% | 3 – 25% | 2.5 – 10% |
| | *Rhodomonas* | 7 – 19% | 8 – 18% | 4 – 9% |
| Haptophyta | *Isochrysis* | 7 – 33% | 1 – 27% | 1 – 40% |
| | *Pavlova* | 7 – 36% | 5 – 29% | 4 – 19% |
| Heterokontophyta | *Nannochloropsis* | 16 – 68% | 9 – 38% | 0.03 – 3% |
| Rhodophyta | *Porphyridium* | 11 – 18% | 2 – 38% | 0 – 0.2% |



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## List of Tables



## Annex Table



## List of Figures





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## Annex Figures





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