This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N° 773330

Deliverable report for

GAIN

Green Aquaculture Intensification

Grant Agreement Number 773330

Deliverable D1.2

Title: Selection of algal strains: preliminary results

Due date of deliverable: 30/04/2019
Actual submission date: 20/05/2019

Lead beneficiary: WU
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WP 1 – Production and Environment
Task 1.1 – Algae as multifunctional feed components

Dissemination Level:		
PU	**Public**	**Y**
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	Y

Document log

Version	Date	Comments	Author(s)
Version 1	10/04/2019	Preliminary	Bárbara Guimarães, Sarah D'Adamo
Version 2	30/04/2019	Updated version from Bela's and Roberto's comments	Bárbara Guimarães, Sarah D'Adamo

Recommended Citation

Guimaraes, B. Barbosa M., Wijffels, R., D'Adamo, S., Report on selection of algal strains: preliminary results. Deliverable 1.2. GAIN - Green Aquaculture INtensification in Europe. EU Horizon 2020 project grant nº. 773330. 24 pp.

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GLOSSARY OF ACRONYMS

Acronym	Definition
CO_2	Carbon dioxide
DW	Dry Weight
EPA	Ecosapentanoic 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
Se [VI]	Selenate
Se [IV]	Selenite
Zn	Zinc

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1. Introduction

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. 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.

In GAIN WP1, Task 1.1, we aim to select macroalgal and microalgal strains which could be used as multi-value fish feed components (Figure 1).

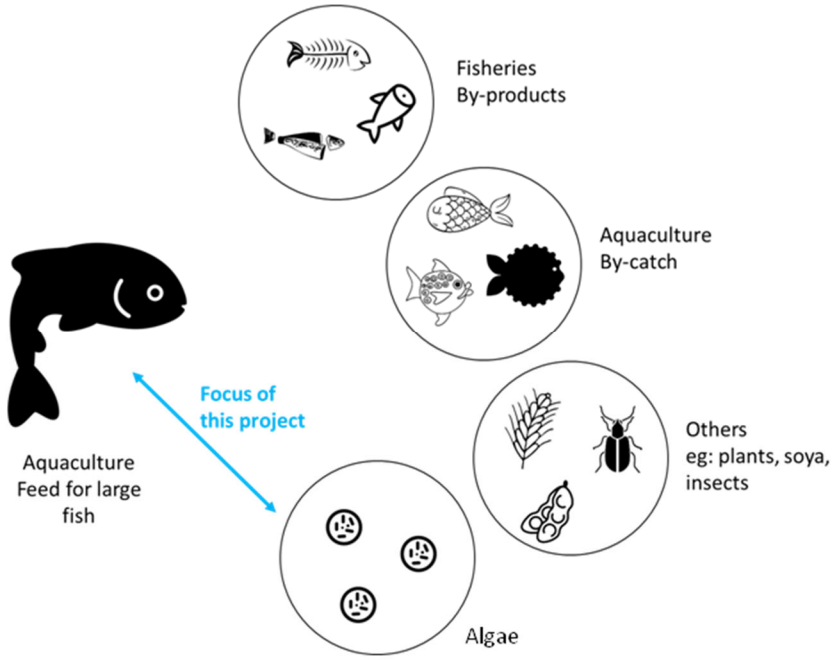

Figure 1 – Ingredients used in fishfeed and the use of algae in this project as a microingredient.

Task motivation

Fish feed plays a major role in aquaculture, representing up to 50% of production costs (Craig and Helfrich, 2002). 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). 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 fish skin and the composition of its body fluids and tissues (Lall and Tibbetts, 2009). Fish formulation is, therefore, very important for the aquaculture industry.

Care must be taken into supplementing the diet with the necessary trace minerals, in order

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to increase nutrient utilisation (Naylor et al., 2000 and 2009) and strengthen fish immune system. A study by Wang showed that antibody production increased as the amount of selenium intake increased (Wang et al, 1997). However, current industrial mineral premixed contain inorganic forms e.g. zinc oxide, zinc sulphate and sodium selenite (Rider et al., 2010), which are not readably absorbed. As a result, copper, zinc and cadmium excretion can contaminate sediment beneath fish farms (Dean et al., 2007). This has led to the implementation of regulatory/maximum levels of mineral concentration in fish feed formulation (Rider et al., 2010). However, these regulatory levels are still below fish dietary requirements. Therefore, there has been an interest in looking for better ways to increase micronutrients their bioavailability (Rider et al., 2010). For example, fish feed containing selenium enriched-yeast proved to be more bioavailable than that including Se in inorganic form – eg. sodium selenite (Rider et al., 2010). The search for more bioavailable organic forms in still ongoing, as Selenium concentration in salmon feeds has been steadily decreasing, as fish meal, which a has a high selenium content, e.g. 1.4 mg kg^{-1} for Atlantic herring (Bryszewska & Måge, 2015), has been progressively substituted by plant derived protein (Berntssen, Amlund, Sele, & Ørnsrud, 2018; Sissener et al., 2013).

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 solve many micro-ingredient challenges in aquaculture. They able to produce and store a variety of compounds of interest such as proteins, lipids, carotenoids and minerals (Borowitzka, 1997), (Shah et al., 2018). These results are promising however, there is still a lack of industrial supply of algae (Simard and le Gouvello, 2017) which poses a challenge for its further implementation in other application within the aquaculture industry.

Macroalgae can also be included as feed ingredient supplying these minerals: in addition they provide binder effects due to sulphated polysaccharides and stimulate fish immune system via beta glucans (Belghit et al., 2017; Biancarosa et al., 2018).

Task aim

Task 1.1 aims to increase the amount of biominerals, namely Zinc and Selenium, in algae that will be incorporated in fish feed whilst ensuring they are bioavailable for the fish. The increase in the amount of zinc and selenium is aimed at positively impacting the fish health, by strengthening the immune system and reducing the likelihood of disease outbreaks. Furthermore, improving the retention efficiency of these elements it is relevant for eco-intensification as it leads to decrease their concentrations in waste sludge of land based fish farms, thus making it possible to reuse the sludge, after drying and sanitation, as fertiliser (see GAIN D2.3 for details). To achieve this aim, Zn concentration in sludge should be reduced from 300-600 mg Kg^{-1} to 150 mg Kg^{-1}. As regards Se we aim to improve its retention efficiency, in order to comply with the nutritional (Khan et al, 2017) and EU regulatory requirements (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R1095&from=EN). Selenium content in animal feed is regulated by the European Union (EU): its concentration in animal feed cannot exceeds of 0.5 mg/kg (EC 1831/2003 and amendments). Furthermore, only 0.2 mg selenium/kg can be added as a supplement (Sele, Ørnsrud, Sloth, Berntssen, & Amlund, 2018). However, according to (Berntssen et al., 2018), the average selenium concentration in fish feed supplemented, either with inorganic selenite or selenized yeast preparation, is about 0.6 mg

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kg^{-1}, as the feed industry tend to satisfy nutritional requirements. Therefore, to comply with regulatory issues and desirable selenium content in salmon feed, selenium compounds with a high bioavailability are required. Macroalgae and microalage may provide such bioavailable selenium compounds via Se enrichment after (Yan, Zheng, Chen, Lin, & Zhang, 2004). As a target for this selenium enrichment, we aim for a minimum concentration of 150 mg Se per kg dry macroalgae. Such seaweeds could be added directly to the fish feed and would not significantly increase the proportion of seaweed in the feed formula.

During the first year, Task 1.1 focused on the selection of macro and microalgae strains. This deliverable describes the preliminary results achieved during the first 12 months. The first step of the approach is to select the most suitable algal strains for fish feed purposes. Algal biomass containing minerals will be supplied during the project to the partners in charge of fish feed manufacture (SPAROS) and feed trials will be performed in Task 1.2 to determine the bioavailability of the minerals in the feed (Figure 2).

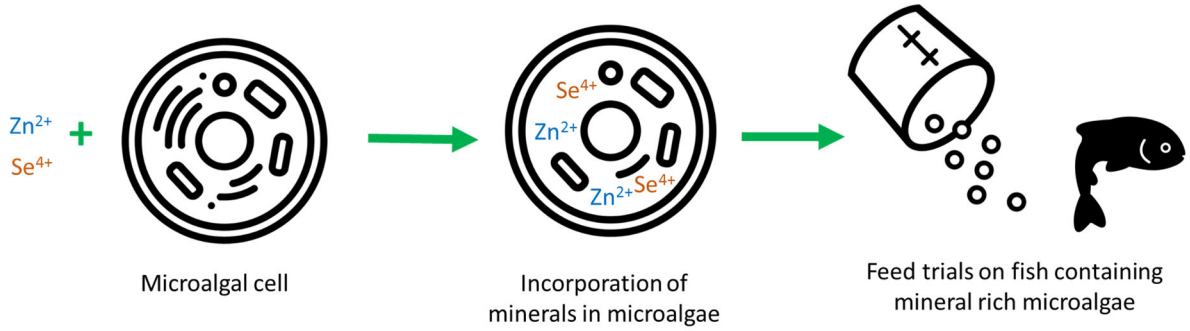

Figure 2 – Outline of mineral incorporation in microalgal biomass, fish feed and used for fish trials.

SPAROS determined Zn and Se target concentrations in microalgae, , assuming 0.5 % microalgae fraction in fish feed.
These values are summarized in Table 1.

Table 1. Target Zn and Se values in fish feed and microalgae dry biomass

Mineral concentration Target in fish feed		Mineral concentration Target in microalgal dry biomass
Zn	180 mg/kg fish feed	36 mg/Kg
Se	0.5 mg/kg fish feed	0.1 mg/Kg

2. Methods

Literature mining and selection criteria

Several considerations were made as pre-selection criteria, based on literature mining.
The selection criteria for the most suitable microalgae species involved:
- robustness (microalgae that have been grown at large scale, are fast growers, and are less sensitive to outdoor fluctuating conditions), industrial relevance and already usage in

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aquaculture.
- reported capability to accumulate metals (i.e. strains used in bioremediation of heavy metals);
- seawater vs freshwater microalgal strains: since fish trials will focus on seabream and seabass, as the most relevant species both in volume and value for the European aquaculture, we set a preference for marine microalgae; 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.
-fish digestibility: strains without or with a weaker cell wall may be preferable for fish meal digestibility;
-presence of other value-added molecules in the biomass, such as 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
- 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.

Macroalgae strains were selected in collaboration with SHP taking into account: (
high contents of minerals required as ingredient in salmon feed;
(II) low contents of undesired minerals (e.g. As, Hg, Cd);
(III) relevant concentrations of sulphated polysaccharides (alginates, carragenans)
(IV) high concentrations of beta glucans,
(V) production costs and availability

Culturing conditions

Media

The four marine microalgae strains mentioned above need to be grown under the same conditions (temperature, CO_2, agitation, irradiance and nutrients) so that the experiments are comparable. The first step was the media formulation. The microalgal medium used consists of artificial seawater enriched with nitrate, phosphates and minerals.
The medium formulation was carefully adjusted to avoid precipitation issues once increasing mineral concentrations. The ionic species containing sulphur (in the original formulation) were substituted by chloride forms in order to easily manipulate sulphur concentrations by means of a single media ingredient (Na_2SO_4). This medium was named chloride medium. Selenium (Se) is not part of most algal cultivation media so it will be individually tested to understand tolerance levels.

Microalgal strains and culturing conditions

Nannochloropsis sp., Dunaliella sp., Tetraselmis sp. and Phaeodactylum tricornutum were ordered from culture collections and grown in the laboratory at the Bioprocess Engineering group at Wageningen University and Research.
These marine strains were cultured in the same media under standard light and CO_2 conditions. Experimental inoculum was grown in 250 mL Erlenmeyer flasks in order to ease the design of experiments.

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Macroalgal (seaweed) cultivation

Saccharina latissima and *Alaria esculenta* were produced after (Buck & Buchholz, 2004). Fertile gametophytes were collected from wild populations and the sori were carefully cleaned with cellulose tissue. Spore release was triggered by overnight incubation at 6+2° C in a moist plastic container, followed by incubation in sterile seawater.. Seedlings were kept on 2 mm Kuralon ropes in tanks at 7.5+2.5° C and subsequently deployed at sea for 3-5 months until harvested. Other seaweeds were harvested from wild populations.

Experimental conditions

Strain robustness screening

Strain robustness was assessed over the period of a week to understand whether the altered media components would have an impact on the cell growth. The four microalgal strains were tested under a basal medium (f/2) and changes were made to vary the ratios and content of the nitrate and phosphate, which are essential for cell division, and trace minerals. The experiment lasted 7 days. Four different conditions were employed, as summarised in Table 2.

Table 21 - Media experimental conditions: N stands for Nitrogen and P for Phosphorus.

Media experimental conditions	Main media components		
	Nitrogen	*Phosphorus*	*Minerals* (micronutrient stock, $FeCl_3$, $ZnCl_2$, Na_2SO_4)
Condition 1	1 x [N]	1 x [P]	1 x [minerals]
Condition 2	1 x [N]	1 x [P]	20 x [minerals]
Condition 3	20 x [N]	20 x [P]	1 x [minerals]
Condition 4	20 x [N]	20 x [P]	20 x [minerals]

The flasks were left for a week and pictures were taken for qualitative information.

Selenium (Se) tolerance

Selenium tolerance was assessed over a period of a month for prolonged exposure. The four microalgal strains were grown in duplicates (n=2) in Erlenmeyer flasks. This was an initial screening and duplicates were used an indicative for follow up experiments. Two different conditions were used:

Condition 1:
f/2 media with basal concentrations of N, P, trace minerals.

Condition 2:
f/2 media containing 2X of zinc and trace concentrations of selenium

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The flasks were left for a month and pictures were taken for qualitative information.

***Nannochloropsis sp*. growth curves**

Nannochloropsis sp. were grown in triplicates (n=3) in Erlenmeyer flasks in chloride media under standard pH, light and CO_2 conditions. The experiment lasted 10 days. Three starting cell concentration were tested: optical density (OD) of 0.25, 0.5 and 0.75. Optical density and quantum yield (QY) were daily monitored.

***Tetraselmis sp*. growth curves**

Tetraselmis sp. were grown in triplicates (n=3) in Erlenmeyer flasks in chloride media under standard pH, light and CO_2 conditions. The experiment lasted 8 days. Two starting cell concentration were tested: OD of 0.5 and 0.75. Optical density and quantum yield were daily monitored.

Correlation curve: Cell Count, OD and Dry weight

Nannochloropsis sp. were grown in triplicates (n=3) in Erlenmeyer flasks in chloride media under standard pH, light and CO_2 conditions. The experiment lasted 24 days. One starting cell concentration was used: OD of 0.25. Optical density, quantum yield and dry weight were daily monitored.

Selenization of macroalgae

Approach for selenium enrichment of macroalgae (seaweeds) has been shown by Yan, Zheng, Chen, Lin, & Zhang, 2004. The approach was tested for the seaweeds *Saccharina latissima, Laminaria hyperborea* and *L. digitata*. Briefly, these macroalgae have been collected from wild populations and were consequently incubated in seawater enriched with 200 mg l-1 sodium selenite.

The optimal selenization temperature was found to be 10+2^{o} C, where a biomass density of 0.1 kg seaweeds per liter seawater could be used. At higher temperatures the seaweeds either degraded quickly or required a much lower biomass density in the selenization tanks.

To determine selenium uptake by the seaweeds, samples were taken in triplicates randomly from different seaweed individuals after 1, 2, 3, 4, 5, 10, 20, 24, 30, 40, 48 and 50 h. The samples were carefully washed with freshwater for 30 seconds and consequently dried at 45+5^{o} C.

Mineral analysis

Equipment

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. The equipment used will be an Inductively coupled plasma atomic emission spectroscopy (ICP-OES), present at Wageningen University.

Macroalgae analysis were carried out, SHP: multielement analysis was peformed by inductively coupled plasma mass spectrometry after microwave assisted wet digestion (Biancarosa et al., 2018).

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Ionic Species

The main ionic species that will be studied through ICP-OES are zinc, selenium and sulphur. Other minerals may also be tested to assess any presence of cadmium or arsenic contamination when using lower grade chemicals at large scale cultivation.

Sample preparation

Supernatant samples are stored in 1 % nitric acid and microalgal biomass is centrifuged, freeze-dried and then processed for ICP-OES analysis.

3. Results and Discussion

Literature mining

A literature mining was conducted to: 1) establish the amount of information available on zinc and selenium accumulation in microalgae,; 2) identify the four most suitable microalgae candidates to be used for fish-feed incorporation, after testing their tolerance for Zn and Se

Most literature information is based on fundamental studies on metal transport or detoxification in model organism such as freshwater *Chlamydomonas* , which are not relevant for the microalgae industry (Wong and Oliveira, 1991; Morlon *et al.*, 2006; Vriens, Behra, Voegelin, Zupanic and Winkel, 2016), (Johnson et al. 2007; Saavedra et al. 2018), or exotic/indigenous species, usually isolated in specific polluted area around the world (Chong, Wong and Tam, 2000; Ying *et al.*, 2012).

We set several constraints to select suitable microalgal species that can be readily used in industrial set-up (TRL5-7), without dealing with further implementation (scale-up optimization) or impending regulations such as Nagoya protocol (https://www.cbd.int/abs/), which may put restrictions on exploitation of isolated strains outside the EU-zone.

The constraints list and the reason behind can be found in the Methods section.

From this mining study, we identified that the most frequently species used in commercial mariculture operations are the diatoms: *Skeletonema costatum, Thalassiosira pseudonana, Chaetoceros gracilis, C. calcitrans,* the flagellated*: Isochrysis galbana, Tetraselmis suecica, Monochrysis luther,* and certain species of *Chlorella spp*. However, most of these species are mainly used for direct feeding mollusc/shrimp larvae, not as fish feed ingredient (Coutteau, FAO report http://www.fao.org/tempref/docrep/fao/003/w3732e/w3732e02.pdf). Moreover, diatoms are difficult to grow in large scale because of their shear stress and low biomass productivity (<0.5 g/l), compared to other microalgal species (Wang and Seibert 2017).

As feed ingredient, instead, omega 3-6 lipid rich *Nannochloropsis*, *Phaeodactylum*, have been considered positively in the fish feed industry, as well as *Tetraselmis* and *Dunaliella* are also considered a good source of starch, protein, and carotenoids such as lutein and beta-carotene, respectively (Tafreshi et al. 2009, Pereira et al. 2019). These 4 microalgal species stood up because of their industrial application and interest, as they are already grown for food and nutraceutical purposes (Hamann and Jacobsen, 2018; Garcia et al, 2017, Camacho et al. 2019).

Moreover, these microalgal 4 species has been also showed to be able to remove metal

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contaminants (including Zn, Se, Cd) in ecotoxicological studies (Torres et al. 2017, Perales-Vela et al. 2006).
Table 3 summarizes the result of the literature mining with respect to the selection criteria.

Table 3 – Summary of the literature mining with respect to microalgaa selection criteria.

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

Strain robustness screening

Strain robustness was assessed to determine which strains were more resistant to changes in media composition, as high concentrations of nutrients can become toxic. As first screening, the studied conditions were used for only qualitative observations. Four nutrient medium concentrations were tested, as described in Section 2, *strain robustness screening*. Condition 1 represents the control medium, and it is based on the nutrients concentration used in the reference medium of f/2 medium. Conditions 2-4 have different ratios and increased concentration of nutrients up to 20X, compared to the original recipe.
In previous experiments in our laboratory, a 20X increase of N and P concentrations showed to promote growth and efficiently increase biomass concentration for certain microalgae species, which is ideal for both lab-scale analyses and final scale-up. Thus, condition 3 assesses and confirm whether the 4 microalgae species can reach higher biomass concentration, using higher N and P concentrations.

In condition 2 only the minerals were increased in order to screen for microalgae species less sensitive to higher mineral concentrations. In condition 4, N, P and mineral concentrations were all increased of 20X and this condition would represent the target medium composition, as ideally it is favourable to reach high biomass concentrations and no growth limitation due to trace mineral limitation/toxicity.

In general, most of the cultures responded better to an increase of 20 fold of all medium components (condition 4) or 20 fold increase of just nitrate and phosphate (condition 3). Moreover, they were all capable of tolerating higher concentrations of minerals (condition 2) and cultures were more pigmented. This highlights the robustness of the strains, and the capability of tolerating high amounts of trace minerals.

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Selenium tolerance

Selenium exposure has never been studied in *Nannochloropsis sp., Dunaliella sp., Tetraselmis sp.* and *Phaeodactylum tricornutum.* Previous studies conducted only on freshwater algal strains, such as *Chlorella pyrenoidosa* (Zhong and Cheng, 2017; Zhao *et al.*, 2019) and *Chlamydomonas* (Vriens, Behra, Voegelin, Zupanic, Winkel, *et al.*, 2016) and *Haematococcus pluvialis* (Zheng *et al.*, 2017), demonstrate very different ranges of selenium tolerance. Thus, it is important to expose the cultures to trace concentrations of selenium to have a preliminary understanding of their selenium tolerance. Moreover, most of the microalgal media do not contain selenium. For this experiment, a trace concentration of Se of 1.97 mg/L was chosen accordingly to literature data available for other species (Zhong and Cheng, 2017; Zhao et al., 2019; Vriens, Behra, Voegelin, Zupanic, Winkel, et al., 2016; Zheng et al., 2017). To assess whether this concentration is suitable also for the 4 microalgal species of interest, a small trial was conducted, monitoring growth at such concentration: the results are shown in Figure 3.

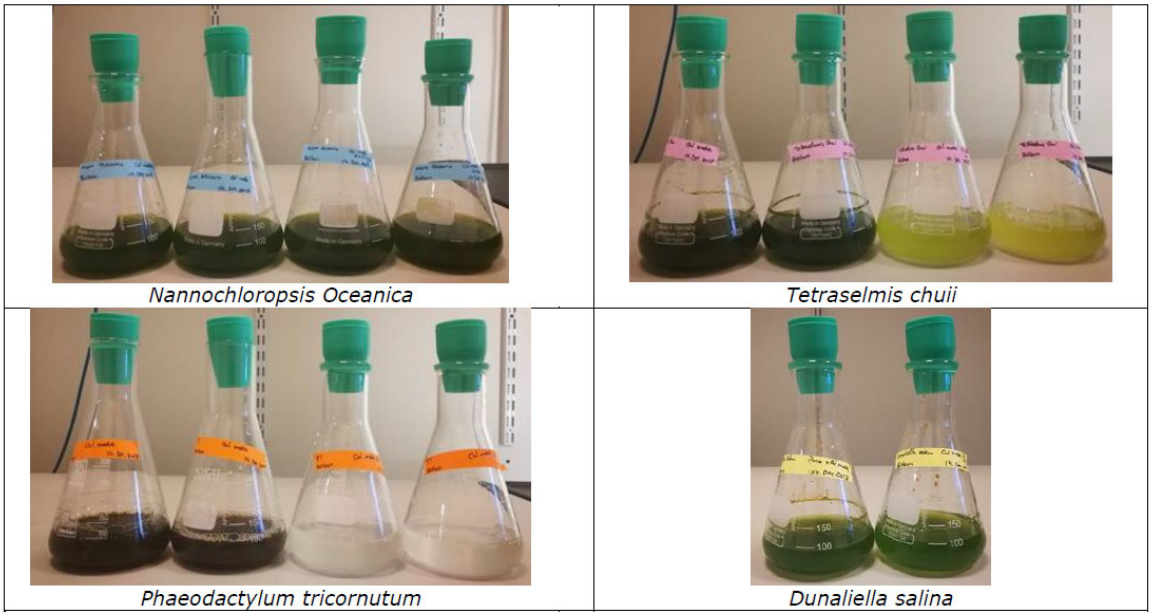

Figure 3. Results from the preliminary qualitative test. Per picture: the left Erlenmeyer flasks are control groups; the right Erlenmeyer flasks contain sodium selenite. The name of the microalgae species is described below the picture.

In each picture of 1 month old cultures the flaks were arranged in the following order:

- Condition 1 and duplicates (n=2)*, Condition 2 and duplicates (n=2)*

**Dunaliella sp.* was also studied in duplicates (n=2) but the picture was only taken of 1 flasks of each condition.

Results showed no visible growth inhibition of *Nannochloropsis sp.* and *Dunaliella sp*. *Tetraselmis sp.* showed an intermediate effect on cell growth and *Phaeodactylum tricornutum* cultures exposed to selenium died.

Biomass accumulation for mineral analysis

After this screening it was decided to continue with strains that visually were not affected or relatively affected by selenium exposure. Thus, *Nannochloropsis sp.* and *Tetraselmis sp.* were

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selected for the following experiments, which involves a protocol development for mineral analysis. Not knowing how much mineral is accumulated in the cells, and how much is extractable, high biomass concentrations are required to perform a preliminary analysis.
It was assessed that for the preliminary mineral analysis a biomass concentration of 3g/L per sample is required. Therefore, an experiment was performed to determine the optimal starting cell concentration to reach high biomass levels in shake flasks. We analysed growth performance in the culturing conditions chosen for both *Nannochloropsis sp.* and *Tetraselmis sp.* As medium composition is changed, it is necessary to assess growth curves and it is ideal to monitor the photosynthetic performance by measuring the fluorescence of chlorophyll by portable fluorometer (AquaPen); a stable QY between 0.68-0.72 is usually sign of healthy state cultures.

Nannochloropsis sp. growth curves

An experiment was performed to determine the optimal starting cell concentration to reach high biomass levels for *Nannochloropsis sp*.
Optical density (OD) was used as a measurement for cell growth and quantum yield (QY) was used an indication of cell viability (Figure 4).

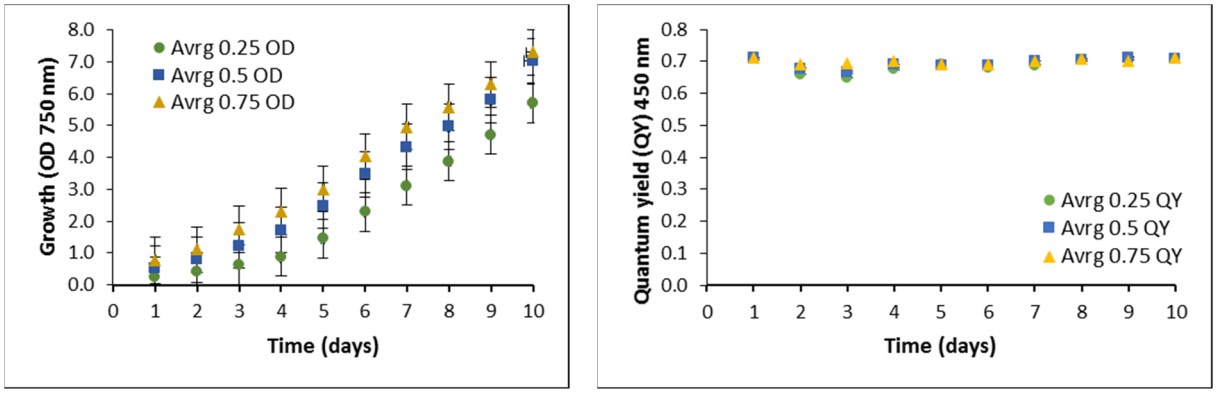

Figure 4 – Cell growth and viability under chosen culturing conditions. From left to right: Cell growth over a period of 10 days. Quantum yield over a period of 10 days.

Tetraselmis sp. growth curves

As above, an experiment was performed to determine the optimal starting cell concentration to reach high biomass levels for *Tetraselmis sp.*
Optical density (OD) was used as a measurement for cell growth and quantum yield (QY) was used an indication of cell viability (Figure 5).

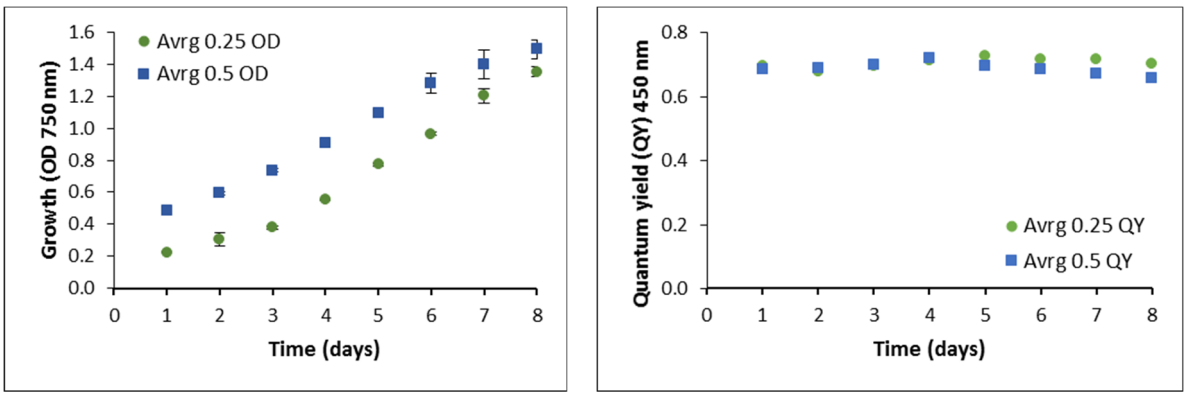

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Figure 5 – Cell growth and viability under chosen culturing conditions. From left to right: Cell growth over a period of 8 days. Quantum yield over a period of 8 days.

The QY values were high and constant during the experiment for both strains (0.7 ± 0.1). This shows that the cells were not stressed under this culturing conditions. However, *Nannochloropsis sp*. reached an OD of up to 7 in exponential growth, while *Tetraselmis sp,* could reach only OD of 1.5, before entering in stationary phase (Figure 11 and 12). Moreover, it was observed that *Tetraselmis sp.* cultures clumped or crashed frequently during the experiments (Figure 6). Thus, *Nannochloropsis sp*. was selected as best strain for further protocol development.

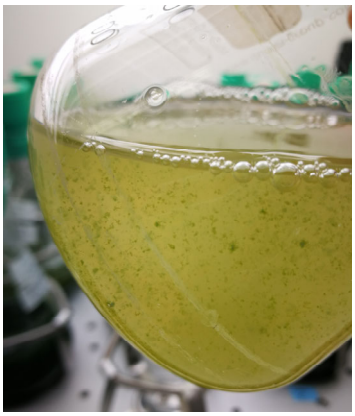

Figure 6 – Picture taken of a *Tetraselmis sp.* culture that clumped after inoculation.

Correlation curve: Cell Count, OD and Dry weight

The aim of this experiment was to look in depth which biomass concentration (g/L) can be reached under these conditions. Moreover, it is ideal to have a correlation curve between OD and dry weight (DW) to facilitate future analyses*. Nannochloropsis sp.* cultures were monitored over a period of 24 days. OD and quantum yield measurements and dry weight were taken to determine cell growth and viability over time (Figure 7, Figure 8).

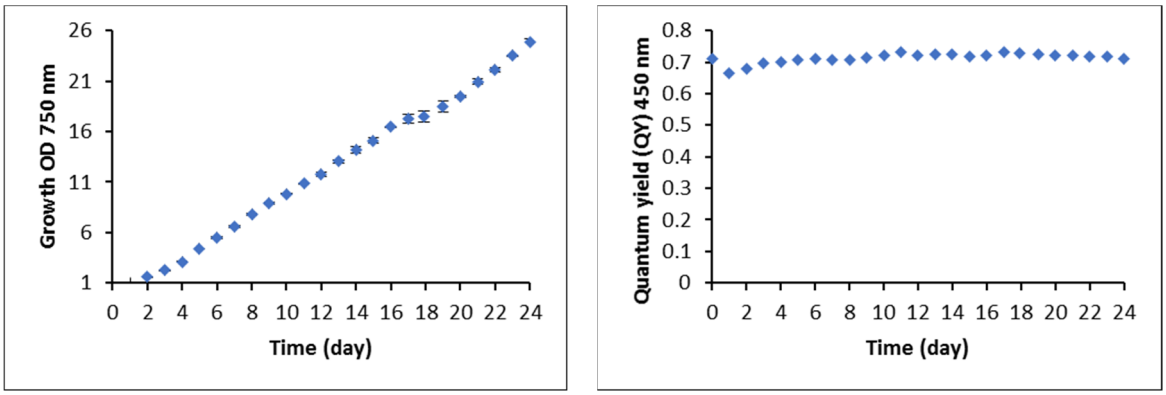

Figure 7 - Cell growth and viability under chosen culturing conditions. From left to right: Cell growth over a period of 24 days. Quantum yield over a period of 24 days.

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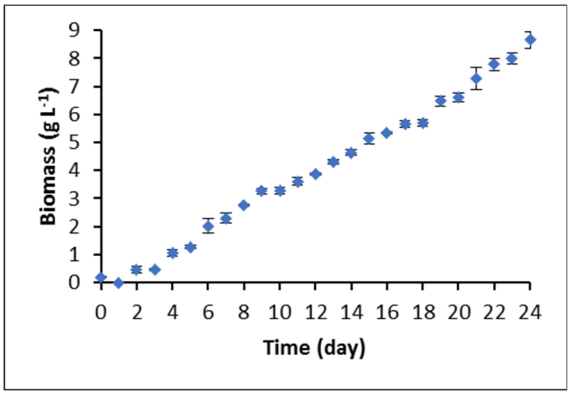

Figure 8 – Biomass concentration (g DW/L) over a period of 24 days.

The final biomass concentration reached was of 8.66 (g DW/L). Biomass productivity was calculated and reached 0.35 g/L/day in shake flasks.
This experiment allowed us to confirm that under the culturing conditions chosen it is possible to reach a biomass concentration suitable for ICP analysis. For the preliminary mineral analysis a biomass concentration of 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. Biomass for ICP analysis was collected in order to determine basal zinc uptake.

ICP-OES method development for microalgal biomass

Macroalgae and microalgae biomass digestion and mineral analysis follow different protocols.
A method for ICP-OES detection of mineral was developed for treating microalgal biomass (Figure 9) and ensuring reproducibility over the different experiments. Cells were harvested by centrifugation,the cell pellets were washed to remove any medium residual, and freeze-dried. The freeze-dried biomass was processed through a microwave-assisted acid digestion; after digestion, these samples were loaded into an inductively coupled plasma optical emission spectroscopy (ICP-OES) machine (PerkinElmer Avio® 5000) to determine total content of interest Zn and Se.

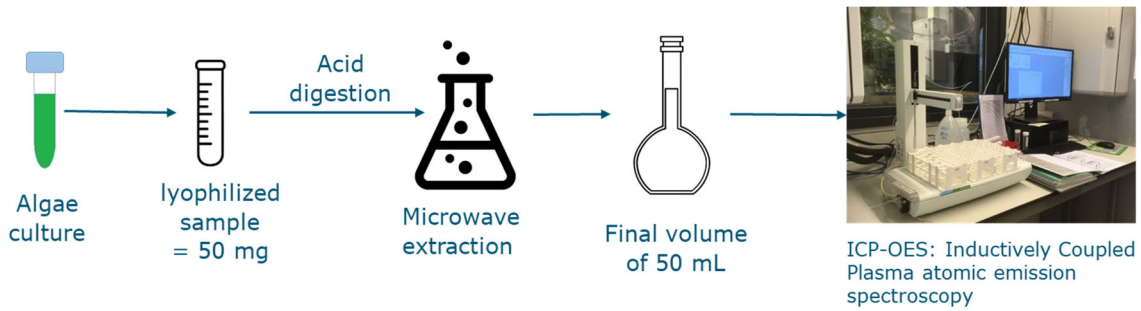

Figure 9: ICP-OES method development for lyophilised algal samples.

Zinc analysis

Preliminary data from biomass samples from *Nannochloropsis* (n=3) indicated zinc values ranging between from 0.06 and 0.07 mg of Zn per kg of dry microalgal biomass. These values were obtained by growing the microalgae at different concentrations of zinc in the

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medium, from standard concentration (0.27 mg/L), normally present in the growth medium as trace element, up to 4.12 mg/L (Figure 10). This basal zinc value from the preliminary analysis is below the target inclusion value. This result suggests that zinc may be used in very small amounts by cells and is not accumulated. The second hypothesis is that free ions of zinc (Zn^{2+}) may be not fully available in the medium, as thet are 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. Therefore, a series of experiments will be performed to increase Zn concentration ranging from: exposure to increasing zinc concentrations, absence and changes of types of buffer and the use of chelating agents such as EDTA that may affect the zinc availability and transport in the cell.

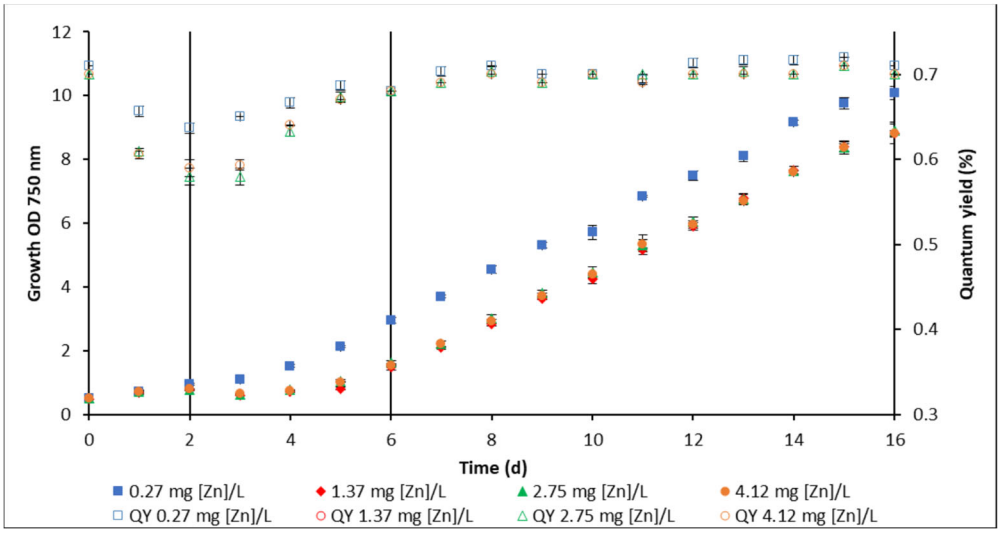

Figure 10 - The growth and quantum yield of the different zinc concentrations measured over 14 days. No visual effect is seen on growth from elevated Zn concentrations in the culture media.

Se analysis

Selenium is not commonly used as trace element in microalgal growth medium, therefore it was important to assess working concentrations. As for Zinc analysis, cells were grown in medium containing different concentrations of Se. The first screening was done in a range of concentrations from 0 (control) up to 3 mg/L. In this range, *Nannochloropsis* does not show growth inhibition and the Se concentration in microalgae biomass was 0.09 ± 0.001 mg of Se per kilo of dry microalgal biomass in Erlenmeyer flasks, which is to the target set for the GAIN project. Further experiments will be conducted with higher ranges of concentration of Se in the medium, in order to understand maximum possible inclusion level, and toxicity thresholds.

Moreover, Se uptake in *Nannochloropis* grown at different Se concentration (0-3 mg/L), indicated that this strain is actively uptaking this mineral in the biomass. A manuscript describing the work will be prepared, because up-to date, very little information is available regarding Se accumulation and uptake in salt water strains.

Mineral content of macroalgae for fish feed ingredients

By using a carefully composed seaweed mixture as fish feed ingredient SHP GAIN partner was able to substitute the following minerals from the fish feed mineral pre-mix: 32% of Mn, 19 % of Fe, 2 % Cu, 9 % Zn, 9 % Ca, 51 % Na, 90 % K, 62 % Mg and 2 % P. After selenization

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screening, it is found out that *S. latissima* accumulated selenium to an average content of 213.33+23.25 mg kg^{-1} after 48 h, whereas *L. digitata* and *L. hyperborea* exhibited a selenium content of 323.33+76.64 mg kg^{-1} already after 5 h incubation (after 48 h: 350+82.87 mg kg^{-1}). These macroalgae can be used during the fish feed composition.

4. Conclusions

From these results, *Nannochloropsis sp* appeared to be the most promising strain in terms of robustness and less toxicity response to both selenium and zinc. We were able to assess that a culturing period of 12 days is ideal to obtain samples with a biomass concentration which allows mineral analysis.

Moreover, the results presented in this document showed that *Dunaliella sp.* and *Tetraselmis sp.* and *Phaeodactylum* were successfully acclimatised to the different media composition, but further optimisation is needed to reach optimal growth conditions for *Tetraselmis sp.*

This report documents preliminary results regarding the selection of microalgal strains. Full details will be reported in forthcoming papers in scientific journals using OpenAcess to ensure optimal dissemination of the results.

The macroalgal analysis carried out by SHP partner shows that macroalgae can be a good mineral source for fish feed, in addition to binder effects and immune system stimulating properties. Selenized seaweeds and microalgae may be a new alternative to selenized yeasts.

5. Future work

Future work will aim at studying uptake and toxicity levels in *Nannocholoropsis sp.* for different zinc concentrations and compounds present in the medium that hinder Zn uptake (buffering and chelating agents). Regarding Se, different concentration ranges and inorganic sources will be tested (Se [VI] Selenate; Se [IV] Selenite). This will be done by performing growth curves under the same conditions, looking into cell viability and performing mineral analysis at the end of the experiment. Zinc and selenium accumulation will be first studied separately, in order to understand the impact of each mineral on the microalgal growth performance. A range of concentrations will be tested for each mineral and samples will be taken for ICP-OES analysis and determination of mineral uptake. In parallel, the other strains that were pre-selected will also be grown under the same conditions and their basal mineral uptake and toxicity will be studied and compared, in order to confirm the preliminary results. Comparative mineral uptake screening of the four strains will result in the selection of the most promising strain for fishfeed trials.

Finally, experiments will focus on simultaneously incorporating both zinc and selenium in the microalgal cell . This will allow for a single stage process for the microalgal biomass growth and lower production costs. Once found the best mineral concentrations for media formulation which allow to obtain the highest mineral accumulation into microalgal biomass, these will be employed for cultivation upscaling (25L, 300L, 1500L) (Figure 11), in order to obtain enough biomass for fish feed trials.

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SPAROS will make feed formulation using the algal biomass provided by SHP and WUR.

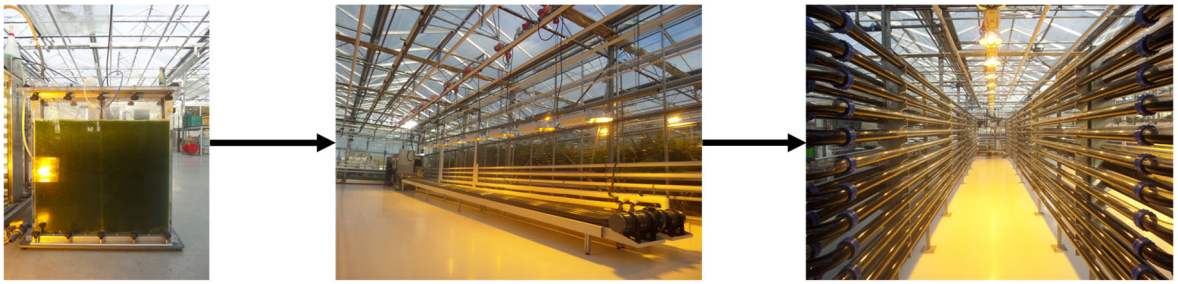

Figure 11 – The scale up procedure of microalgae biomass for the fish feed trails on the different reactors available at AlgaePARC from the 25 Liter, to 300 to 1500 litre system. These systems will run during 2020 to achieve the necessary amount for the fishfeed trials.

References

AFD, EU and GIZ (2017) Opportunities and challenges for aquaculture in developing countries. Available at: https://europa.eu/capacity4dev/hunger-foodsecurity-nutrition/documents/opportunities-and-challenges-aquaculture-developing-countries.

Becker, E. W. (2013). Microalgae for Aquaculture: Nutritional Aspects. In Handbook of Microalgal Culture: Applied Phycology and Biotechnology. https://doi.org/10.1002/9781118567166.ch36

Belghit, I., Rasinger, J. D., Heesch, S., Biancarosa, I., Liland, N., Torstensen, B., Bruckner, C. G. (2017). In-depth metabolic profiling of marine macroalgae confirms strong biochemical differences between brown, red and green algae. Algal Research, 26, 240-249.

Berntssen, M., Amlund, H., Sele, V., & Ørnsrud, R. (2018). Evaluation of the maximum limits for selenium in Atlantic salmon feeds. Rapport fra havforskningen.

Biancarosa, I., Belghit, I., Bruckner, C. G., Liland, N. S., Waagbø, R., Amlund, H., Lock, E. J. (2018). Chemical characterization of 21 species of marine macroalgae common in Norwegian waters: benefits of and limitations to their potential use in food and feed. Journal of the Science of Food and Agriculture, 98(5), 2035-2042.

Borowitzka, M. A. (1997). Microalgae for aquaculture: Opportunites and constraints. Journal of Applied Phycology.

Bryszewska, M. A., & Måge, A. (2015). Determination of selenium and its compounds in marine organisms. Journal of Trace Elements in Medicine and Biology, 29, 91-98.

Buck, B. H., & Buchholz, C. M. (2004). The offshore-ring: A new system design for the open ocean aquaculture of macroalgae. Journal of Applied Phycology, 16(5), 355-368. doi:10.1023/B:JAPH.0000047947.96231.ea

Camacho F et al. (2019) Potential Industrial Applications and Commercialization of

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

Microalgae in the Functional Food and Feed Industries: A Short Review Mar. Drugs 2019, 17(6), 312; https://doi.org/10.3390/md17060312

Chong, A. M. Y., Wong, Y. S. and Tam, N. F. Y. (2000) 'Performance of different microalgal species in removing nickel and zinc from industrial wastewater', Chemosphere. Pergamon, 41(1–2), pp. 251–257. doi: 10.1016/S0045-6535(99)00418-X.

Craig, S. and Helfrich, L. A. (2002) Understanding Fish Nutrition, Feeds, and Feeding, Virginia Cooperative Extension. doi: https://pubs.ext.vt.edu/420/420-256/420-256.html.

European Commission, D. (2017) THE EU HIGHLIGHTS THE EU IN THE WORLD EU MARKET SUPPLY CONSUMPTION TRADE EU LANDINGS AQUACULTURE PRODUCTION. European Union. Available at: http://www.eumofa.eu/documents/20178/108446/The+EU+fish+market+2017.pdf/80acad95-907f-4b90-b2a7-1086964df3d9 (Accessed: 17 July 2018).

Garcia JL et al. (2017) Microalgae, old sustainable food and fashion nutraceuticals. Microbial Biotechnology, 10, 1017–1024.

Hamann and Jacobsen, 2018 Microalgae for Salmon Feed https://www.food.dtu.dk/-/media/Institutter/Foedevareinstituttet/Publikationer/Pub-2019/Rapport-Microalgae-for-Salmon-Feed-Supply-Chains-Markets-and-Sustainability.ashx?la=da&hash=994D03CEE755675757666B3AC5F2B94D952D691C.

Johnson HL et al. (2007) Copper and Zinc Tolerance of Two TropicalMicroalgae After Copper Acclimation. Environmental ToxicologyDOI 10.1002/tox.20265

Krężel A and Maret W (2016) The biological inorganic chemistry of zinc ions. Arch Biochem Biophys. 2016 Dec 1; 611: 3–19. doi: 10.1016/j.abb.2016.04.010

Lall, S. P. and Tibbetts, S. M. (2009) 'Nutrition, Feeding and Behaviour of fish', Veterinary Clinics of North America Exotic Animal Practice, 12(2).

Lucas, J. S. and Southgate, P. C. (2012) Aquaculture : farming aquatic animals and plants. Wiley-Blackwell. Available at: https://www.wiley.com/en-us/Aquaculture%3A+Farming+Aquatic+Animals+and+Plants%2C+2nd+Edition-p-9781405188586 (Accessed: 15 August 2018).

Moomaw W., Berzin I. and Tzachor A. (2017): Catalyzing Innovation – Cutting out the middle fish: Marine Microalgae as the Next Sustainable Omega-3 Fatty Acid and Protein Source; in Industrial Biotechnology, vol 13, no. 5, October 2017, pp234-243

Morlon, H. et al. (2006) 'Selenite transport and its inhibition in the unicellular green alga Chlamydomonas reinhardtii', Environmental Toxicology and Chemistry, 25(5), pp. 1408–1417. doi: 10.1897/2512039.1.

Mutter R. (2013): Algae – Hope or Hype? In IntraFish.com, July 2013

Naylor, R. L. et al. (2000) 'Effect of aquaculture on world fish supplies', Nature, 405(6790), pp. 1017–1024. doi: 10.1038/35016500.

Naylor, R. L. et al. (2009) 'Feeding aquaculture in an era of finite resources.', Proceedings of the National Academy of Sciences of the United States of America. National Academy of Sciences, 106(36), pp. 15103–10. doi: 10.1073/pnas.0905235106.

Perales-Vela HV et al. 2006 Heavy metal detoxification in eukaryotic microalgae

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

Chemosphere 64 (2006) 1–10

Pereira H et al. (2019) Nutritional Potential and Toxicological Evaluation of Tetraselmis sp. CTP4 Microalgal Biomass Produced in Industrial Photobioreactors. Molecules 2019, 24, 3192

Rider, S. A. et al. (2010) 'Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (Oncorhynchus mykiss) diet', Journal of Animal Physiology and Animal Nutrition. Wiley/Blackwell (10.1111), 94(1), pp. 99–110. doi: 10.1111/j.1439-0396.2008.00888.x.

Roy S, Pal R (2015) Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics Proc Zool Soc (2015) 68(1):1–8 DOI 10.1007/s12595-013-0089-9

Saavedra R. et al. 2018 Comparative uptake study of arsenic, boron, copper, manganese and zinc from water by different green microalgae. Bioresource Technology Volume 263, September 2018, Pages 49-57

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H., & Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. Journal of Trace Elements in Medicine and Biology, 47, 124-133.

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaeimehr, A., … Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. Journal of Applied Phycology, 30(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Shields, R. J., & Lupatsch, I. (2012). Algae for Aquaculture and Animal Feeds. Technikfolgenabschätzung – Theorie Und Praxis 21., 21(1), 23–37.

Simard, F. and le Gouvello, R. (2017) Sustainability of Fish Feed in Aquaculture: Reflections and Recommendations CONTENT. Paris. doi: https://doi.org/10.2305/IUCN.CH.2017.02.fr.

Simard, F., & le Gouvello, R. (2017). Sustainability of Fish Feed in Aquaculture: Reflections and Recommendations CONTENT. Paris. https://doi.org/https://doi.org/10.2305/IUCN.CH.2017.02.fr

Sissener, N., Julshamn, K., Espe, M., Lunestad, B., Hemre, G. I., Waagbø, R., & Måge, A. (2013). Surveillance of selected nutrients, additives and undesirables in commercial N orwegian fish feeds in the years 2000–2010. Aquaculture Nutrition, 19(4), 555-572.

SPAROS Lda. (2014) Feed Ingredients in Aquaculture: A Database of Aquaculture feed ingredients. Available at: http://www.sparos.pt/images/BOOKLET2.pdf (Accessed: 13 July 2018).

Tafreshi AH and Sharati M (2009) Dunaliella biotechnology: methods and applications Journal of Applied Microbiology10714–35

Torres EM et al. (2017) Impact of inorganic contaminants on microalgae productivity and bioremediation potential. Ecotoxicology and Environmental Safety 139 (2017) 367–376368 http://dx.doi.org/10.1016/j.ecoenv.2017.01.034

Towers L (2013) https://thefishsite.com/articles/the-use-of-algae-in-fish-feeds-as-alternatives-to-fishmeal

Vriens, B., Behra, R., Voegelin, A., Zupanic, A. and Winkel, L. H. E. (2016) 'Selenium Uptake

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

and Methylation by the Microalga Chlamydomonas reinhardtii', Environmental Science and Technology. doi: 10.1021/acs.est.5b04169.

Wang J and M Seibert(2017) Biotechnol Biofuels. 2017; 10: 16. Prospects for commercial production of diatoms. doi: 10.1186/s13068-017-0699-y

Wang, C., Lovell, R. T. and Klesius, P. H. (1997) 'Response to Edwardsiella ictaluri Challenge by Channel Catfish Fed Organic and Inorganic Sources of Selenium', Journal of Aquatic Animal Health. Wiley-Blackwell, 9(3), pp. 172–179. doi: 10.1577/1548-8667(1997)009<0172:RTEICB>2.3.CO;2.

Wong, D. and Oliveira, L. (1991) 'Effects of selenite and selenate on the growth and motility of seven species of marine microalgae', Canadian Journal of Fisheries and Aquatic Sciences, 48(7), pp. 1193–1200. doi: 10.1139/f91-144.

Yan, X., Zheng, L., Chen, H., Lin, W., & Zhang, W. (2004). Enriched accumulation and biotransformation of selenium in the edible seaweed Laminaria japonica. J Agric Food Chem, 52(21), 6460-6464.

Ying, G.-G. et al. (2012) 'Biosorption of zinc and copper from aqueous solutions by two freshwater green microalgae Chlorella pyrenoidosa and Scenedesmus obliquus', Environmental Science and Pollution Research. doi: 10.1007/s11356-012-0800.

Zhao, Y. et al. (2019) 'Toxic effect and bioaccumulation of selenium in green alga Chlorella pyrenoidosa', Journal of Applied Phycology. Springer Netherlands, 31(3), pp. 1733–1742. doi: 10.1007/s10811-018-1711-z.

Zheng, Y. et al. (2017) 'Effects of selenite on green microalga Haematococcus pluvialis: Bioaccumulation of selenium and enhancement of astaxanthin production', Aquatic Toxicology, 183, pp. 21–27. doi: 10.1016/j.aquatox.2016.12.008.

Zhong, Y. and Cheng, J. J. (2017) 'Effects of Selenite on Unicellular Green Microalga Chlorella pyrenoidosa: Bioaccumulation of Selenium, Enhancement of Photosynthetic Pigments, and Amino Acid Production', Journal of Agricultural and Food Chemistry. American Chemical Society, 65(50), pp. 10875–10883. doi: 10.1021/acs.jafc.7b04246.

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ANNEX 1 - GAIN Consortium and project structure

Table A 2 - GAIN Consortium

Participant № (leadership role)	Participant legal name	Country	Type
1 (Coordinator; WP5; WP7)	Università Ca' Foscari Venezia (UNIVE)	Italy	RTD
2 (WP3)	The University of Stirling (UoS)	UK	RTD
3 (WP1)	Alfred-Wegener-Institut Helmholtz- Zentrum für Polar- und Meeresforschung (AWI)	Germany	RTD
4	IBM Ireland Limited (IBM)	Ireland	CORP[1]
5 (WP2)	Agencia Estatal Consejo Superior de Investigaciones Cientificas (CSIC)	Spain	RTD
6 (WP4)	Longline Environment Limited (LLE)	Ireland	SME
7 (WP6)	Sparos Lda (SPAROS)	Portugal	SME
8	Salten Havbrukspark (SHP)	Norway	SME
9	Wageningen University (WU)	Netherlands	RTD
10	Johann Heinrich von Thuenen-Institut, Bundesforschungsinstitut Fuer Laendliche Raeume, Wald Und Fischerei (TI)	Germany	RTD
11	Agrifood and Biosciences Institute (AFBI)	UK	RTD
12	Zachodniopomorski Uniwersytet Technologiczny W Szczecinie (ZUT)	Poland	RTD
13	Asociacion Nacional de Fabricantes de Conservas de Pescados y Mariscos-Centro Tecnico Nacional de Conservacion de Productos de la Pesca (ANFACO)	Spain	NPO[2]
14	Multivector AS (MV)	Norway	SME
15	Gildeskal Forskningsstasjon AS (GIFAS)	Norway	SME
16	Lebeche (LEBCH)	Spain	CORP[1]
17	Sagremarisco-Viveiros de Marisco Lda (SGM)	Portugal	SME
18	Fondazione Edmund Mach (FEM)	Italy	NPO[2]
19	Dalhousie University (DAL)	Canada	RTD
20	South China Sea Fisheries Research Institute (SCSFRI)	China	RTD

GAIN is structured in 7 Work Packages, plus an Ethics Work Package, which was added by the EC during the negotiation (see Figure 12). WP leaders are indicated in Table A 2. The main objects of each WP are listed below.

WP1 - Production and Environment: will develop novel sustainable feeds and tools for enhancing aquaculture sustainable management of aquafarm based on Big Data analytics.

WP2 - Secondary products: will develop new co-products, in order to enhance circularity, sustainability and profitability of aquaculture supply chains.

WP3 - Policy and markets will analyse the state-of-the-art of EU and national legislations with respect to the valorisation and marketing of innovative GAIN products and co-products and provide suggestions to policy makers.

WP4 - Eco-intensification: will develop new approaches and tools for assessing the level of eco-intensification of GAIN innovative solutions, in comparison with standard practices.

[1] Corporation (Not SME)

[2] Non-profit Organisation

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WP5 - Professional development: will deliver both on-line and in presence courses, in order to facilitate the adoption of GAIN innovative solutions by aquafarm operators.

WP6 - Dissemination, Exploitation, Communication: will maximize GAIN impact, by careful matching communication&dissemination tools to targeted audiences and developing platforms for exploiting GAIN results beyond its life time.

WP7 - Coordination: will ensure the timely delivery of all GAIN contractual items.

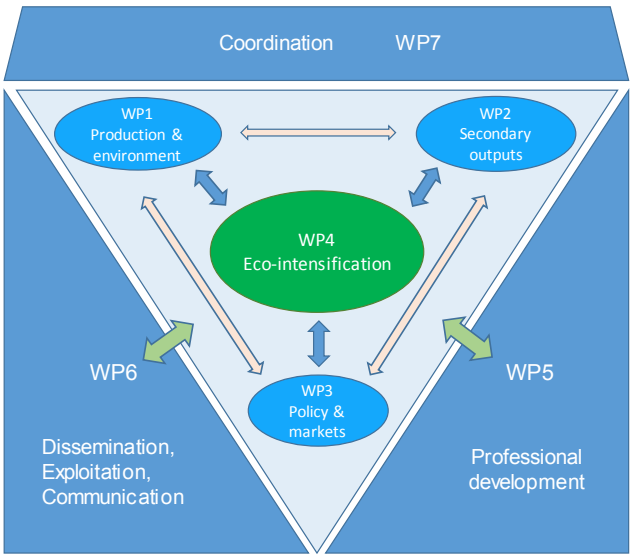

Figure 12 GAIN structure

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