Evaluate the use of retail food waste for the production of *Chlorella vulgaris*

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Master's in Functional Biology and Plant Biotechnology
Faculty of Sciences of the University of Porto
2023

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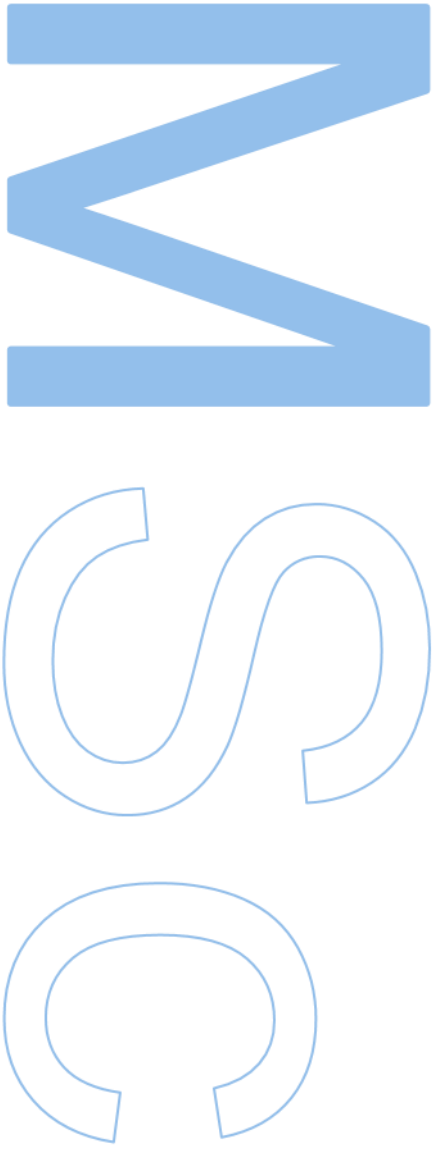


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Dedicated to all those who have always given me the strength to carry on

Acknowledgements

Reservo este espaço para expressar a minha gratidão pelo contributo que várias pessoas tiveram, para que este trabalho fosse possível. Este trabalho contou com o apoio de enumeras pessoas, do Porto ao Algarve, que ao longo destes 11 meses contribuíram com todo o esforço, tempo e dedicação.

Inicialmente, pouco sabia sobre microalgas e no final desta etapa, sinto que evoluí não só como pessoa, mas também profissionalmente. Em primeiro lugar, tenho de expressar uma enorme gratidão à Dra. Helena por me ter ajudado imenso neste trabalho com as suas sugestões, apesar de eu ter sido de vez em quando “cabeça dura”. Também tenho a agradecer por me ter aceitado como estagiário numa empresa, onde vim conhecer pessoas que levarei para sempre no coração. Deixo também um agradecimento especial à Prof. Dra. Paula Melo, com quem sempre adorei trabalhar e que apesar da distância, mostrou-se sempre disponível para me ajudar, disponibilizando-se também com ideias e recomendações.

De uma forma especial, deixo um enorme agradecimento aos meus amigos que foram um grande apoio psicológico e me deram muita força para nunca desistir dos meus objetivos. Não esqueço a família que deixo em Pataias, nem todo o conhecimento que partilharam e que proporcionou para que eu evoluísse. Um especial agradecimento à Inês Guerra, “Minichefe”, e ao Gonçalo Espírito Santo, o meu Padrinho, que foram como uns mentores para mim. Tiveram, sem dúvida, muita paciência para as minhas mil e uma perguntas, e que tornaram esta etapa mais agradável do que stressante.

Por fim, aos meus pais e familiares, que com muito esforço financeiro, me deram a oportunidade de fazer um curso superior e me apoiaram incondicionalmente. A muitos outros que não referi, mas que também foram muito importantes na concretização desta etapa, um muito obrigado!

“A maior gratidão que eu pude mostrar foi pessoalmente que resultou nas memórias que irão guardar de mim!”

Resumo

O desperdício alimentar pode nunca ser eliminado e, dado o problema atual associado ao crescimento da população mundial, pode tornar-se muito pior. Há uma crescente consciencialização e preocupação com o desperdício alimentar e cada vez mais estudos se concentram em encontrar soluções para este problema. Quando os alimentos atingem um estado inadequado para o consumo humano, tornam-se resíduos e acabam em aterros, onde contribuem para as emissões de gases com efeito de estufa, através da digestão anaeróbica, com impacte direto nas alterações climáticas. Em resposta a esta questão, as microalgas, como a *Chlorella vulgaris*, podem contribuir estrategicamente para mitigar as emissões de CO_2 e reciclar os nutrientes dos resíduos alimentares numa matéria-prima versátil. Este trabalho aborda este problema, "redirecionando" os alimentos que não podem ser vendidos ou doados pelos retalhistas para o meio de crescimento, que representa aproximadamente 20% dos custos de produção das microalgas. Assim, os nutrientes são reciclados para alimentar as microalgas e obtém-se um produto valioso, enquanto os custos globais de produção são reduzidos e, em última análise, o impacte ambiental negativo dos resíduos alimentares é atenuado, contribuindo para a economia circular.

Em primeiro lugar, foram analisados sete tipos de resíduos para avaliar as suas diferentes composições e selecionar os mais promissores para serem utilizados como meio de cultura para *C. vulgaris*, com base na concentração de nutrientes essenciais. Foram testadas estratégias para melhorar a disponibilidade de nutrientes e a estabilidade dos resíduos, considerando a viabilidade em ambiente industrial. Finalmente, a formulação otimizada do meio foi utilizada para escalar *C. vulgaris* e avaliar o seu potencial no processo global, em contexto industrial.

Palavras-chave: Meio de cultura; *Chlorella vulgaris*; Redução do desperdício alimentar; Economia circular

Abstract

Food waste may never be eliminated, and given the current problem associated with global population growth, it could become much worse. There is increasing awareness and concern about food waste, and more studies are focusing on finding solutions to this problem. When food reaches a state which is unsuitable for human consumption, it becomes waste and ends up at landfill sites, where it contributes to greenhouse gas emissions, from anaerobic digestion, directly impacting climate change. In response to this issue, microalgae, such as *Chlorella vulgaris*, can contribute strategically to both mitigate CO_2 emissions and upcycle food waste nutrients into a versatile feedstock. This work addresses this problem, by redirecting food that cannot be sold or donated by retailers, into growth medium, which accounts for approximately 20% of microalgae production costs. Thus, nutrients are recycled to feed microalgae and a valuable product is obtained, while overall production costs are reduced. Ultimately the negative environmental impact of food waste is mitigated, contributing to a circular economy.

First, seven waste types were screened to assess their different compositions and to select the most promising one to be used as a culture medium for *C. vulgaris*, based on key nutrient concentrations. Strategies to improve nutrient availability and residue stability over time were tested, considering the feasibility in industrial settings. Finally, the optimized medium formulation was used for scaling up *C. vulgaris* and assessing the potential of the overall process in an outdoor industrial setting.

Keywords: Culture medium; *Chlorella vulgaris*; Reduce food waste; Circular economy

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List of Abbreviations [if applicable]

FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
UP	UNIVERSITY OF PORTO
DW	DRY WEIGHT
OD	OPTICAL DENSITY
PBR	PHOTOBIOREACTOR
rpm	ROTATIONS PER MINUTE (r/min)
m	MASS
R^2	CORRELATION COEFFICIENT
p-value	SIGNIFICANCE VALUE
µ	SPECIFIC GROWTH RATE
SD	STANDARD DEVIATION
t	TIME
T	TEMPERATURE
Prod	PRODUCTIVITY
Org	ORGANIC COMPANY CULTURE MEDIUM
Ino	INORGANIC COMPANY CULTURE MEDIUM
CTL	CONTROL TREATMENT
N	NITROGEN SUPLEMENTATION
Fe	IRON SULUTION SUPLEMENTATION
M	MICRONUTRIENTS SOLUTION (INORGANIC GROWTH)
MB	MICRONUTRIENTS SOLUTION (ORGANIC GROWTH)
MF	MICRONUTRIENTS (INORGANIC GROWTH) AND IRON SOLUTION

1. Introduction

1.1. The problem of food waste and the ZeroW Project

Food waste is a global problem and tends to increase with population growth (Melikoglu, Lin and Webb, 2013) (Figura 1). With increasingly easier access to food in developed countries, people can purchase more products than ever before, and suppliers offer a wide variety to choose from. Therefore, people might buy more products than they consume, and the demand for retailers to provide a choice of fresh products, leads to a need to renew and replace products at a fast pace. Ultimately, this leads to expired foods which can no longer be consumed, either in households or sold by retailers. Some of these products considered unsuitable to be sold can sometimes be donated if they are edible. Otherwise, for instance, if they pose a health risk to consumers, they are disposed of and sent to landfills (Brancoli, Rousta and Bolton, 2017).

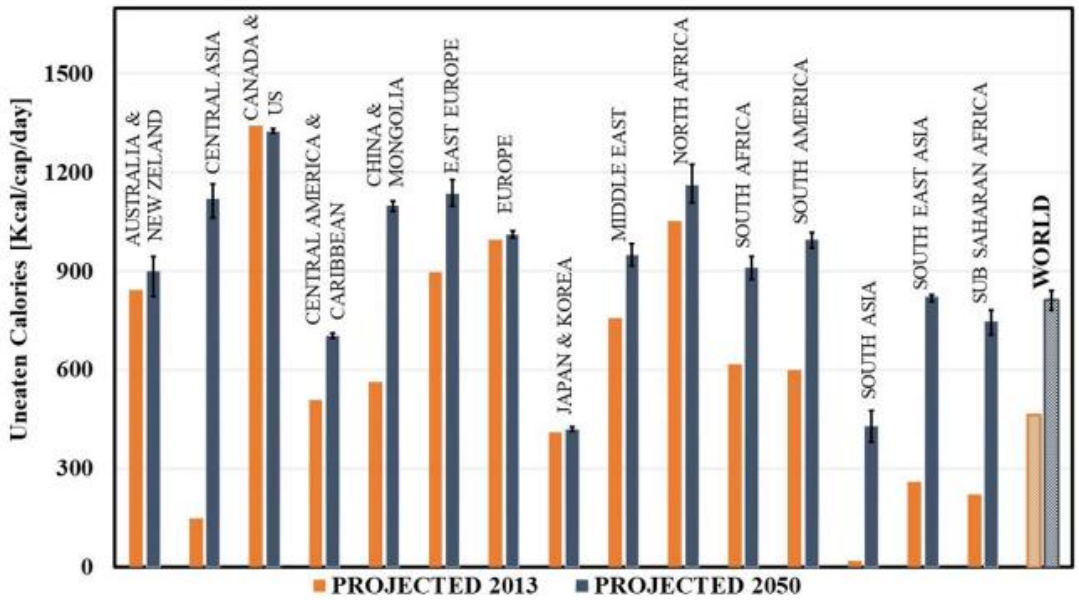


Figure 1: Projected uneaten calories 2013 *and towards 2050* in different countries *(Barrera and Hertel, 2021).*

Ultimately, this path leads to the overgrowth of landfills and consequent negative impacts on the environment caused by the emission of greenhouse gases and contamination of the occupied soil and ground waters (El-Fadel, Findikakis and Leckie, 1997; Bhatia et al., 2023). Another concern that can be taken into account is related to the large amounts of food unnecessarily produced and transported, which also increase emissions, water

consumption, and other impacts on biodiversity (Schmidt and Matthies, 2018). Regarding the food that is still in a good state to be consumed, even if it is not appealing to the consumer in some cases, this food can still be uptaken. For instance, associations are working with supermarkets to collect and distribute food to those in need, and some organizations repurpose “ugly foods” into other consumables, as is the case of fresh produce turned into juice.

As food waste is inevitable and has been increasing with population growth, it is urgent to find solutions to repurpose it. There has been a growing concern to minimize waste and find new sustainable strategies to counteract the risk of eutrophication caused by discarded high phosphorus and nitrogen content (Kolev Slavov, 2017). The scientific community has therefore been looking for eco-friendly strategies to minimize this problem, beyond physical and chemical treatment of waste (Kolev Slavov, 2017). The use of food waste is gaining more prominence because it consists of around 60 % carbohydrates, 20 % proteins and 10 % lipids (Li et al., 2013), making it a valuable raw material for recovering nutrients needed in many biotechnological processes and for producing high-value products (Sayeki et al., 2001). As microalgae have become a topic of interest in the scientific community, more and more studies have emerged where industrial waste is reused as a culture medium, in an effort to reduce cultivation costs.

In response to this complex problem, the Zero Waste EU project arises (https://www.zerow-project.eu/). In ZeroW different strategies to reduce food waste are tested under 9 different living labs. In living lab #8 Allmicroalgae, U. Minho, and SONAE established a partnership to test the viability of applying microalgae to reduce food waste.

1.2. Benefits and potential of microalgae

Within the scientific community, microalgae are often described as photosynthetic organisms that could be eukaryotic (green algae) (Ng et al., 2015)or prokaryotic (cyanobacteria)(Richmond and Hu, 2013). Microalgae and cyanobacteria already have been considered as one group only, by sharing a large number of characteristics (Pulz, Scheibenbogen and Groß, 2001; Udayan, Arumugam and Pandey, 2017).

There are over 50,000 known species of microalgae, but only 30,000 have been studied, analysed and classified, distinguishing themselves mainly by pigmentation, life cycle and basic cell structure (Richmond, 2004). In the microalgae world, the most important classes, in terms of abundance, are Bacillariophyceae (diatom), Chlorophyceae (green

algae), Cyanophyceae (blue–green algae) and Chrysophyceae (golden algae) (Khan et al., 2009; Schmitz, Magro and Colla, 2012; Schmidt and Matthies, 2018).

These photosynthetic microorganisms are found mostly in aquatic freshwater and marine environments but can also be found in terrestrial ecosystems. In the presence of sunlight, they combine water with essential nutrients and atmospheric carbon dioxide to produce biomass (Ng et al., 2015) (Ng et al., 2015), from which valuable molecules can be extracted, such as pigments (carotenoids, powerful antioxidants), proteins and hydrocarbons (Suganya et al., 2016; Siddiki et al., 2022). Microalgae can uptake organic matter and toxic metals from effluents and waste. It is also important to highlight that microalgae produce more oxygen than all the plants in the world combined and are responsible for at least 60% of the Earth's primary production, with faster growth, using less land area and fixing CO_2 more efficiently than complex plants (Ng et al., 2015; Chisti, 2018). These characteristics make microalgae highly interesting and sought-after biotechnological tools (Figure 2).

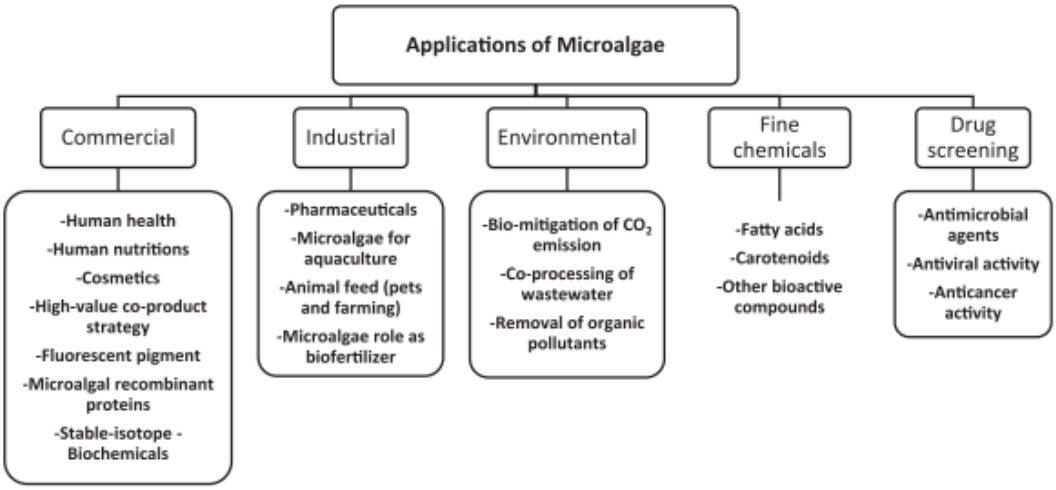


Figure 2: Applications of microalgae in different fields *(Rizwan et al., 2018).*

Microalgae have thus become the focus of many studies in recent years given their great applicability in the food and pharmaceutical industries, in biomedicine, and environmental areas, among others.

1.3. Microalgae industrial production

The conditions for optimum growth vary depending on the species and the cultivation mode. Still, there are common nutritional needs: nitrogen (N) and phosphorus (P) are very important macronutrients. Besides these nutrients, a source of carbon (C) is also required (Grobbelaar, 2004), assimilated through photosynthesis. The depletion of any of these could compromise the assimilation of others (Flynn, 1990).

Phototrophic algae assimilate inorganic carbon (carbon source), in the chemical form of H_2CO_3, HCO^{3-} and CO_2^-, and use light as an energy source (de Carvalho et al., 2019). The assimilation of inorganic carbon by microalgae, converting it into organic matter in the presence of light (photosynthesis), leads to the oxidation of water, releasing O_2 (Richmond and Hu, 2013).

Nitrogen is fundamental to the synthesis of amino acids and consequently proteins. It can be found in the nitrogenous bases of nucleotides, and it is also crucial for the transmission of genetic information, and essential for metabolic processes. Regarding a nitrogen source, most microalgae are capable of assimilating urea, nitrates (NO_3^-), and ammonia (NH_4^+) (Chen et al., 2017). Phosphorus is also an essential macronutrient and represents an important component of nucleic acids and of phospholipid biosynthesis and participates in the modification of protein function and energy transfer(Moseley and Grossman, 2009). Phosphorus is usually assimilated as orthophosphate (PO_4^{-2}) by microalgae (Procházková et al., 2014).

In addition to macronutrients, micronutrients also play an important role: iron (Fe), sodium (Na), boron (B), copper (Cu), manganese (Mn), zinc (Zn), molybdenum (Mo), cobalt (Co) and silicon (Si) (Quigg, 2016). Although they are required in low concentrations (depending on the species), they play an important role in enzymatic reactions and in the biosynthesis of compounds (Delilah et al., 2022). For instance, magnesium is at the core of the chlorophyll molecule.

In addition to nutritional conditions, external factors such as light, temperature, pH, and salinity are important for optimum growth and may cause different impacts, depending on the species (Richmond and Hu, 2013; Rai, Gautom and Sharma, 2015; Serra-Maia et al., 2016).

1.3.1. Microalgal metabolic regimes and production systems

The growth of microalgae can be described as (photo)autotrophic, heterotrophic, or mixotrophic depending on the utilization of a source of carbon and energy to synthesize organic substances through photosynthesis (Subhash et al., 2017). In autotrophic cultivation, microalgae produce biomass by assimilating inorganic CO_2 with light as energy input. While heterotrophic mode requires an organic carbon source to metabolize with no need for light. Mixotrophic cultivation is a mode in which both autotrophic and heterotrophic metabolisms contribute, and both an inorganic or organic carbon can be assimilated, in the presence of light, or depending on fluctuating environmental conditions (Li et al., 2019). The choice of trophic mode is dependent on the species, but also takes into account the cost of the land, energy and water consumption, nutrient requirements and the climate to which algae will be submitted (Borowitzka, 1992).

For the autotrophic production of microalgal biomass, there are two main types of systems, namely open and closed reactors (Murphy and Allen, 2011). An open pond system, such as a raceway, generally benefits from direct access to sunlight, and low construction and operation costs (Bux, 2013). On the other hand, closed systems such as tubular photobioreactors (PBRs), are more complex, but present a lower risk of contamination and evaporative losses and show better control of growing conditions (Mata, Martins and Caetano, 2010; Narala et al., 2016).

Microalgae growing heterotrophically are produced axenically, in closed-stirred reactors, namely fermenters, Figure 3 (Vuppaladadiyam et al., 2018). In this way of production, contamination should be avoided altogether and tighter control over cultivation parameters is possible. Notably, higher cell densities can be achieved since light penetration is not a concern, which allows much higher concentrations of biomass in less time.(Jin et al., 2020). However, this mode of cultivation requires high fixed and variable production costs and yields a final product with a lower concentration of pigments and proteins (relative to the autotrophic), which might decrease the value of the biomass (Barros et al., 2019).

Figure 3: Different microalgae cultivation systems from Allmicroalgae (Pataias). *(*A) Open pound system 200 m^3; (B) Closed bioreactors 90 m^3 (PBR); (C) Industrial 5 m^3 Fermenter.

1.3.2. The potential of *Chlorella vulgaris*

The unicellular eukaryotic green microalga of the species *C. vulgaris* was first isolated by Beijerinck in 1890 (Ahmad et al., 2020). The origin of its name *Chlorella vulgaris* derives from the Greek name “chloros” which means green and the Latin suffix “ella”, which means small size. The specific restrictive “vulgaris” means common or vulgar, however, this term might not fully capture its significant potential (Safi et al., 2014). *Table 1* describes the taxonomic classification of the species *Chlorella vulgaris*.

Table 1: Taxonomic classification of *Chlorella vulgaris,* according to the *World Register of Marine Species (WoRMS, 2023).*

Kingdom	**Plantae**
Division	Chlorophyta
Class	Trebouxiophyceae

Order	Chlorellales
Family	Chlorellaceae
Genus	Chlorella
Specie	*Chlorella vulgaris*

This microscopic microalga, Figure 4, has an ovoidal shape with a diameter of 2 to 10 µm depending on its growth phase (Yamamoto et al., 2004), it also presents structural elements similar to those of plant cells (Safi et al., 2014). This unicellular microorganism is non-flagellated, grows in freshwater and has the capacity to grow in autotrophic, mixotrophic, and heterotrophic conditions. In autothotrophy the optimum pH and temperature range between 7 and 8 and between 25 ºC and 27 ºC, respectively. *C. vulgaris* is widely recognized for its high growth rate, high protein content and high production of lipids and polysaccharides (Arad and Richmond, 2004; Safi et al., 2014; Ng et al., 2015).

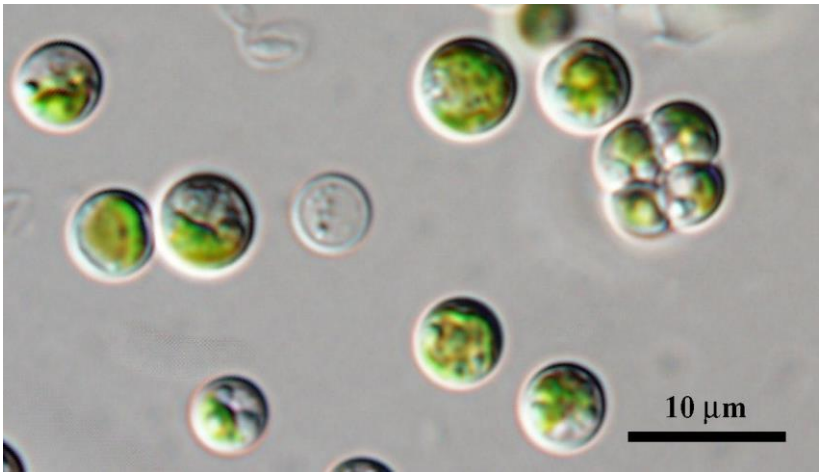


Figure 4: Microscopic view of *C. vulgaris*.

Due to its versatility, adaptability to thrive in different cultivation systems, and the overall value of its biomass, this species presents itself as important and with great potential for several biotechnological applications. For instance, it is commonly used as a food supplement for human and animal consumption (Safi et al., 2014). From a nutritional point of view, *Chlorella* is nutritionally rich, containing over 60% protein, 10 % fat, 15% carbohydrates, trace elements, and vitamins (B complex, thiamine, C, D, E and K) (Rodriguez-Garcia and Guil-Guerrero, 2008; Blas-Valdivia et al., 2011). In recent years

microalgal studies have gained more attention and the consumption of *Chlorella vulgaris* has been reported to prevent or reduce the impact of several metabolic-related diseases, and also to have antiviral (including anti-HIV), antibacterial, and anti-tumor properties (Shibata and Sansawa, 2006).

Currently, besides food and feed, there are numerous applications of this microalga such as for the production of biodiesel feedstock or for biological carbon dioxide sequestration (Felix et al., 2019). Taking into account the intended end-application, it is possible to manipulate the growth of the microalga and to induce it to produce essential compounds for an intended application (Chia et al., 2013). For example, under nitrogen limitation, *C. vulgaris* accumulates lipids, suitable for biodiesel production rather than protein, desired for food development (Rajanren and Ismail, 2017).

There are several large-scale microalgae production systems, however, their production entails high fixed costs. To overcome this issue, several approaches have been proposed, such as the usage of industrial effluents. However, not all effluents are compatible with the production of microalgal biomass for food, pharmaceutical, or cosmetic industries, where there are high quality and safety standards to uphold (Chia et al., 2013).

1.3.3. Large scale: closing the sustainability gap

In theory, the best solution would be to stop food waste at its origin, however, this is not a realistic scenario, but rather much more complex than it may seem. Despite strategies adopted by supermarkets to reduce food waste, better shopping and household practices would also be necessary. At the moment, a single strategy would not be effective enough, so different approaches should be proposed and tested, to have significant results and impacts (Schmidt and Matthies, 2018).

Based on the available literature and previous studies performed at Allmicroalgae, microalgae could be used as a possible answer to this problem. Most of the strategies proposed in the literature focus on solving this issue in the consumers' houses instead of developing strategies to make an impact at a larger scale at the level of supermarket chains and large companies (Aschemann-Witzel, Giménez and Ares, 2018). Many studies suggest positive results on using industrial waste from large companies as a culture medium for microalgae (Cheirsilp, Suwannarat and Niyomdecha, 2011; de Medeiros et al., 2020). To make this strategy viable, however, the composition of the waste needs to be suitable to support microalgae growth, and contain the main nutritional

components necessary, namely nitrogen and phosphorus, at reasonable levels and in a bioavailable form. Many studies have demonstrated that most algae species such as *Scendesmus* sp. (Chinnasamy et al., 2010; Mata et al., 2012), *Chlorella* sp. (Chinnasamy et al., 2010; Feng, Li and Zhang, 2011; Kothari et al., 2012) and *Chlamydomonas* sp. (Chinnasamy et al., 2010; Wu et al., 2012) can be effective tools for bioremediation of industry wastewater effluents. Moreover, these studies also suggest these waste streams to be promising for microalgal biomass cultivation purposes. For example, *Chlamydomonas* sp. was shown to grow in different wastewater from different industries (Chinnasamy et al., 2010; Kothari et al., 2012; Wu et al., 2012).

Furthermore, studies developed at Allmicroalgae, demonstrate it is possible to use organic wastes, for instance, those from the dairy industry, and successfully grow marine microalgae (for instance *Nannochloropsis oceanica*) and freshwater microalgae (such as *Chlorella vulgaris* and *Scenedesmus obliquus*), while also reducing overall production costs (Allmicroalgae, 2019). These results suggest it is possible to achieve competitive productivity using organic wastes as media, compared to the standard industrial formulations. The study performed by de Carvalho et al. shows that it is not only possible to use waste as an alternative organic medium for microalgae growth but also to promote bioaccumulation of lipids, carotenoids, and proteins (de Carvalho et al., 2019).

On the other hand, according to results obtained from SONAE's associated supermarkets, the amount of food destined for donation, mainly bread and cereals, is much less than the amount of food designated as improper for human consumption. According to data obtained from SONAE 2021 reports, food products that are usually unfit for human consumption, and consequently wasted, are mainly meat, fish, crustaceans, mollusks, vegetables, and fruits (SONAE, 2021).

The idea behind reusing waste from companies and retailers as culture media for microalgae can present as a solution to redirect supermarket food waste that would otherwise be sent to landfills. This way, besides repurposing the wasted nutrients and upcycling them into valuable biomass, landfill ground and water contamination may be reduced/avoided to an extent. Therefore, the main goal of the work proposed here is to evaluate the feasibility of using and upcycling nutrients from these wastes to grow *C. vulgaris* and characterize the resulting biomass. Overall, standard working routines of microalgae production are considered and compared to an alternative production pipeline. In particular, a medium preparation and characterization step will be planned, and tested, to assess the industrial viability of growing *C. vulgaris* in a food-waste residue. This working routine is then compared to standard practices at large-scale

microalgae production facilities, with the ultimate goal of producing valuable biomass at a lower cost, while also minimizing the negative environmental impact of food waste.

1.4. Specific aims

The main objective of this work is to develop a protocol to repurpose food waste coming from retailers, which can no longer be donated. This waste is composed of several residues with different proportions of food waste types (meat, fish, bread, dairy products, fruits and vegetables), which are dehydrated and mixed, at a SONAE retail store. These residues were first evaluated to determine which are more suitable to formulate a culture medium. Nutrient availability and different combinations of residues, rich in nutrients, were tested as was their ability to be properly stored until further application. Ultimately, a medium was formulated and characterized, and used to cultivate microalgae, focusing on *Chlorella vulgaris*. The alternative medium solution is further optimized to promote *C. vulgaris* growth and productivity, compared to a standard industrial medium. Finally, the alternative medium is validated at outdoors industrial conditions.

2. Materials and methods

The following work experiments were performed at Allmicroalgae``s facilities, between 12th September 2022 and 28th August 2023.

2.1. *Chlorella vulgaris* standard growth conditions

The *Chlorella vulgaris* strain used for this work was obtained from Allmicroalgae`s culture collection. The base medium used for culture maintenance was an inorganic culture medium that the company recurrently uses, formulated based on to Guillard's F/2 (Andersen, 2005), with nitrates as a nitrogen source at 10 mM of concentration, supplemented with an iron solution at 25 µM and a specific micronutrient solution. This culture was grown starting at the inoculum room of Allmicroalgae`s facilities in 5 L reactors under constant irradiance provided by LEDs (300 µmol photons/m^2/s) at an ambient temperature of approximately 25 ºC. Aeration was attained by compressed air injection through 0.2 µm filters, and coupled with an automated CO_2 injection system, to maintain the pH values between 6,5 and 8.

2.2. Culture medium preparation from waste

The waste provided by SONAE, which had been previously dehydrated, was dissolved in water for 16 hours on a magnetic stirring hotplate (VWR® Advanced) at room temperature at speed 2. After homogenizing the medium was left to sediment without agitation for 24 hours at room temperature without direct sunlight.

Upon separation from the precipitate, the supernatant was decanted into a new sterile Schott flask and filtered with a coffee filter (Continente), Figure 5.

After filtering the homogenised waste, the pH and the salt concentration of the solution was measured with a digital pH meter and a sweater refractometer HI 96822.

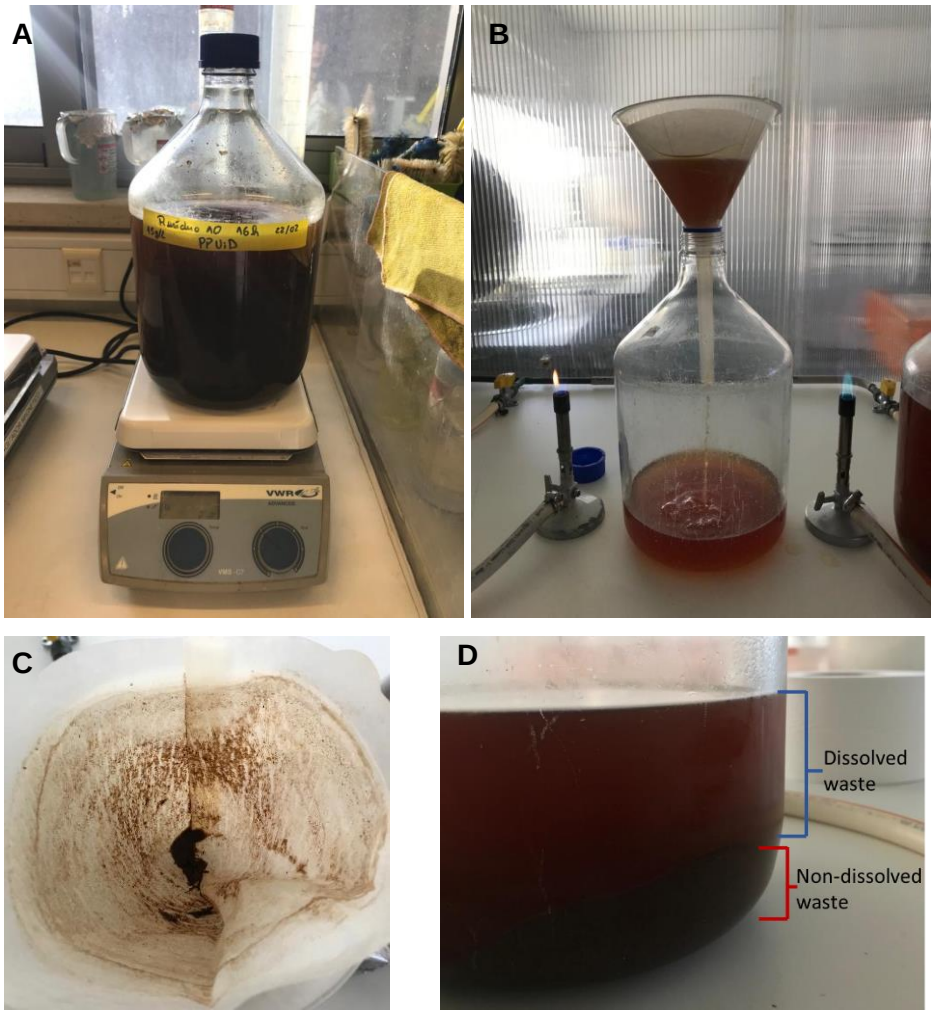


Figure 5: Waste filtration system (A) Schott flask on a magnetic stirring plate, VWR® Advanced, Magnetic hot plates. (B) The system is used to filter out small particles that have not dissolved in the prepared culture medium. (C) Small particles are retained in the coffee filter (D) Culture medium with the residue dissolved in water without stirring after 24 hours, showing part of the Waste that did not dissolve.

2.3. Inoculation and growth assessment

Before inoculation, all materials necessary were sterilized by autoclave, at 121 ºC for 40 minutes. Schott flasks of 0.5 or 1 L were used as the laboratorial assay reactors. All trials were performed in triplicates and samples were collected under sterile conditions whenever necessary.

Microalgal biomass growth was assessed by optical density (*OD*) at 600nm, with a Genesys 10S UV-VIS spectrophotometer Figure 6 (Thermo Scientific, Waltham, MA, USA), by dry weight (*DW*). And by cell counting with a Neubauer chamber, according to the manufacturer`s procedure.

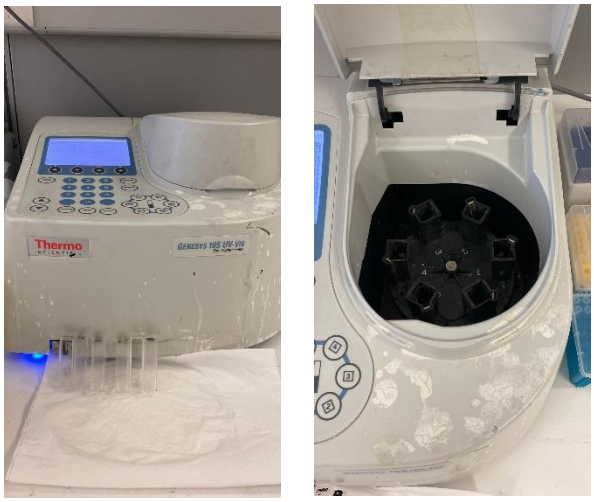


Figure 6: UV/vis Genesys 10S spectrophotometer with quartz cuvettes in front of it (left) and its interior (right).

When inoculating the microalgae, assays started at a concentration of estimated dry weight of 0.35 g/ L (in the inoculation room) or 0.45 g/ L (outdoor trials). Before being used in the inoculum room, the inoculum was previously washed to remove any residual medium. The estimated dry weight was obtained from the OD of the sample, according to the calibration curve (Figure 1, Appendix B):

$$DW_{\,estimated} = 0.3695 * Abs_{\,600\,nm} + 0.11209$$

The real dry weight of the culture was obtained by filtering a known volume of culture in a 0.7 *µm* glass microfiber filter (VWR). After filtering in the vacuum system, the filter was collected to be dried and weighed using a Kern DBS moisture analyzer, Figure 7. According to the following equation, it is possible to determine the concentration of the culture under analysis.

$$DW_{\,real}\,(g\,L^{-1}) = \frac{filter\ weight_{\,final} - filter\ weight_{\,initial}}{Volume_{\,sample}}$$

Figure 7: Kern DBS moisture analyser (left) and its interior (right).

Throughout the trials, both global and maximum productivities were evaluated and calculated as the ratio of the difference in biomass concentration in the end and beginning of the assay by the correspondent time. Global productivity (*Prod $_{global}$*) differs from Maximum productivity (*Prod $_{Max}$*) due to the fact that the latter takes into account only the exponential phase of growth in the test and not the whole growth curve. The following equations were used to calculate both productivities, where DW_B and DW_A represent, respectively, the cellular concentration at the end and beginning of the exponential phase and t_B and t_A are the times, in days, corresponding to those concentrations.

$$Prod_{\,global}\ (g\ L^{-1}Day^{-1}) = \frac{DW_{\,final} - DW_{\,initial}}{t_{\,final} - t_{\,initial}}$$

$$Prod_{\,Max}(g\ L^{-1}Day^{-1}) = \frac{DW_B - DW_A}{t_{\,B} - t_{\,A}}$$

The specific growth rate (μ) was calculated for the batch-grown cultures following the next equation, where DW_B and DW_A represent, respectively, the cellular concentration at the end and beginning of the exponential phase and t_B and t_A are the times, in days, corresponding to those concentrations.

$$\mu\ (Day^{-1}) = \frac{\ln(DW_B - DW_A)}{t_B - t_A}$$

Contaminations were monitored by regular microscopic observations, assessing the culture's viability. A Zeiss® Axio Scope A1 coupled to a ZEN Axicam 503 colour camera was used, Figure 8. To capture and edit the images, the Zen Blue 2.5 lite software was also used.

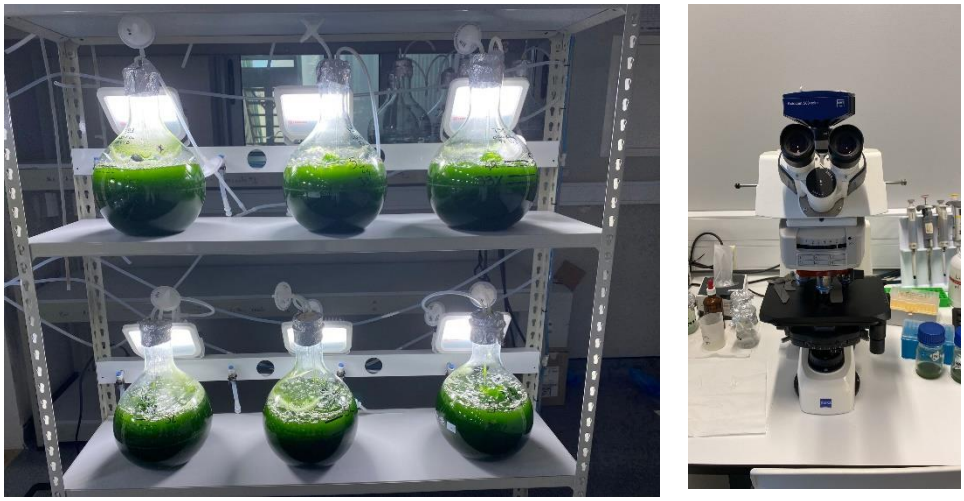


Figure 8: First step of the scale-up of the culture for industrial production at the inoculum room with 5 L bioreactors (Allmicroalgae, Pataias) (left); Zeiss® Axio Scope A1 coupled with a ZEN Axicam 503 colour camera (right).

2.4. Nutritional analyses

Samples of the trials were collected in 15 mL tubes and centrifuged for 10 min at 3500 rpm in a Hermule Z400K centrifuge, Figure 9. The supernatant was collected to quantify nitrates, urea, ammonium, phosphate, iron, and magnesium concentrations.

Figure 9: Hermule Z400K centrifuge *(left) and its interior (right)*

2.4.1. Macronutrient analyses

2.4.1.1. Nitrates quantification

For the nitrates' quantification (adapted from Armstrong (Armstrong, 1963)), the collected supernatant was diluted at a ratio of 1:80, with the addition of 300 µL of hydrochloric acid (HCl, 1 M), for a final volume of 10 mL. The absorbance samples were measured at 220 nm and 275 nm in a UV-Vis Genesis 10S spectrophotometer.

The absorbance reading at 275 nm corresponds to the interference of the organic matter and it was corrected by subtracting it two times from the absorbance reading at 220 nm, as the nitrate ion absorbs UV at 220 but not at 275 nm. The final absorbance was compared to the calibration curve (Figure 2, Appendix B), to obtain the nitrate concentration.

2.4.1.2. Urea quantification

The urea concentration was determined according to the method of Roijers and Tas (Roijers and Tas, 1964). To a diluted sample of 2 mL of supernatant 500 µL urea reagent was added (4 g/ 100 mL of p-dimethylamino benzaldehyde and 4 mL/ 100 mL of sulfuric acid, with ethanol as solvent). The absorbance was measured at 418 nm after 5 minutes on reaction and further compared to a calibration curve (Figure 3, Appendix B).

2.4.1.3. Ammonia quantification (sera ammonium/ammonia-Test (NH_4/NH_3))

Ammonia concentration was determined through dilution of the sample and using an *ammonium/ammonia sera test* Figure 10, according to the manufacturer's recommendations. The absorbance was measured at 697 nm and further compared to a calibration curve (Figure 4, Appendix B).

2.4.1.4. Phosphate quantification (*sera phosphate-Test (PO_4)*)

According to the *phosphate sera test*'s (Figure 10) recommendations, phosphate concentration was determined through proper dilution of the supernatant samples. The absorbance was measured at 716 *nm* and further compared to a calibration curve (Figure 5, Appendix B).

2.4.2. Micronutrient analyses

2.4.2.1. Iron quantification (*sera iron-Test (Fe)*)

Iron concentrations were quantified according to the indications of the sera iron Test (Figure 10) by using a sample of diluted supernatant. The wavelength 561 nm was used to measure the iron and the absorbance was measured using a UV-Vis Genesis 10S spectrophotometer and further compared to a calibration curve (Figure 6, Appendix B).

2.4.2.2. Magnesium quantification (sera magnesium-Test (Mg))

Magnesium sera Test (Figure 10) were used to determine magnesium concentrations on supernatant samples. The magnesium concentration is measured by the number of drops added, according to the indications of the sera test and further compared to a calibration curve (Figure 7, Appendix B).

Figure 10: Aquarium Sera Kits used to quantify the concentration of phosphate, ammonia, iron and magnesium.

2.5. Outdoor validation trials in bubble columns

The outdoors growth assay was performed in 5 L bubble columns, with triplicates (Figure 11). The inoculum used in this test was previously grown in a flat panel photobioreactor (150 L). The inoculum was used once the nitrogen and phosphate source had been completely consumed by the microalgae. Before beginning the assay, the bubble columns were cleaned with concentrated detergent and remained with sodium hypochlorite (100 ppm) until usage. The water and all the solutions used were sterilized (autoclaving) or filtered at 0.2 µm.

The desired pH of the culture was kept under 8, by CO_2 injection in the culture system. The temperature was controlled with a sprinkler irrigation system turned on at temperatures above 30 °C. The compressed air introduced in the bubble columns was filtered at 0.2 µm.

Figure 11: Bubble columns with each specific nutrient solution, prepared to receive the inoculum (right). Bubble columns' final appearance after receiving the inoculum (left).

2.6. Analytical determinations

Liquid culture samples were used for pigment analysis. The samples for this analysis were stored in the freezer at -18 ◦C.

For the biochemical analysis of the other compounds evaluated the biomass obtained at the end of the validation assays was collected and centrifuged at 3500 rpm for 15 minutes. The pellets obtained from biomass were frozen and stored at -18 ◦C.

The biochemical composition of the frozen pellets was evaluated at MarBiotech research group (UAlg) facilities. Before the analysis, the biomass was freeze-dried in a CHRIST Alpha 1-2 L Dplhus, Figure 12, to obtain the powder used for all the needed analyses.

Figure 12: Samples after being freeze-dried (left) and a freeze-dryer, CHRIST Alpha 1-2 L Dplhus (right).

2.6.1. Pigments quantification

For pigment extraction a volume of the liquid culture containing at least 10 mg of biomass was pipetted into a glass tube covered with aluminum to protect from sunlight. Thereafter, the samples were centrifuged at 4500 g for 10 minutes at Hermule Z400K centrifuge and the supernatant was discarded. The pellet was resuspended in 6 mL of acetone and zirconia beads were added (2.5%(v/v) of total volume). Samples were homogenized by vortex (Velp Scientifica Classic Advanced) for 10 min at 1200 rpm and centrifuged at 3500 rpm for 10 min at 4 ºC and then the supernatant was collected (Figure 13). These extraction steps were repeated by adding 6 mL of acetone until the pellet lost color as demonstrated in Figure 13.

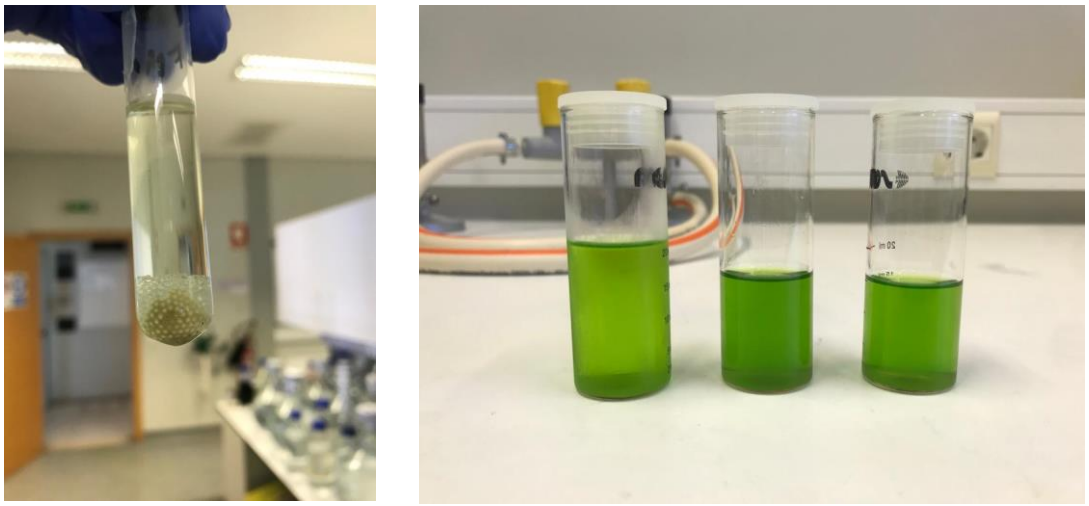


Figure 13: Sample in its final extraction with the pellet without colour (left). The supernatants resulting from the various extractions were collected in a new glass tube (right).

The pigments extracted in the supernatant collected from the various extractions were analyzed by *Genesys 10SUV-VIS* spectrophotometer (*Thermo Fisher Scientific*) in a scanning spectrum from 380 to 700 nm for the determination of chlorophylls (a and b) and carotenoids content. The spectrum corresponding to each pigment was deconvoluted from all data and the corresponding content was calculated using a mathematic model developed by *Allmicroalgae* for that purpose.

2.6.2. Protein content

The protein content was determined by elemental analysis of carbon, hydrogen and nitrogen achieved with a Vario EL III. The biomass was weighed in specific aluminium caps (1-2 mg) in a Sartorius precision balance, according to the procedure provided by the manufacturer. Total protein content was estimated by multiplying the N obtained by 6.25 (Nunez and Quigg, 2016).

Figure 14: Sartorius precision balance used to weigh the aluminium capsules with the sample.

2.6.3. Total lipids content

The crude fat (total lipids) is determined by the modified gravimetric method (Bligh and Dyer, 1959). To the lyophilized biomass (30 mg) 1 mL of chloroform, 2 mL of methanol and 0.8 mL of distilled water were added, followed by homogenization with an IKA Ultra-Turrax disperser on ice for 60 s. Thereafter, 1 mL of chloroform was added, and the mixture was again homogenized for 30 s. Finally, 1 mL of distilled water was added and homogenized for 30 s. Afterwards, the mixture was centrifuged at 4500 rpm for 10 min for phase separation (Figure 15). The organic phase (lower layer) was transferred to a clean tube with a Pasteur pipette, and later a known volume of chloroform (0.7 mL) was pipetted to a pre-weighed tube (wi). The tubes were then placed in a dry bath at 60 °C to evaporate the chloroform (Figure 15).

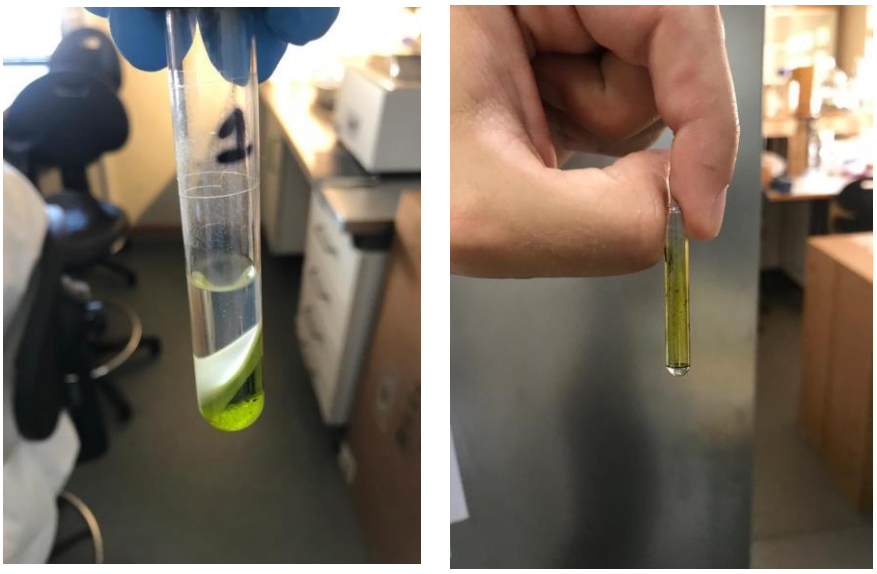


Figure 15: Images of the method for analyzing total lipids. On the left the different phases of the sample after being centrifuged. On the right a glass tube with an organic phase transferred after evaporating the chloroform.

The resulting dried residue was weighed (w_f) in a *Sartorius* precision balance and the percentage of lipids in each sample was calculated according to the following formula:

$$\%\ total\ lipids = \frac{\frac{(\mathrm{wf} - \mathrm{wi}) * 2}{0.7}}{\text{sample weight}} * 100$$

2.6.4. Ashes content

Total ash was determined by the weight difference before and after burning the produced biomass in a muffle. Biomass was weighed and placed in small aluminium cups and burned for 5 h at 550 °C using a furnace Figure 16.

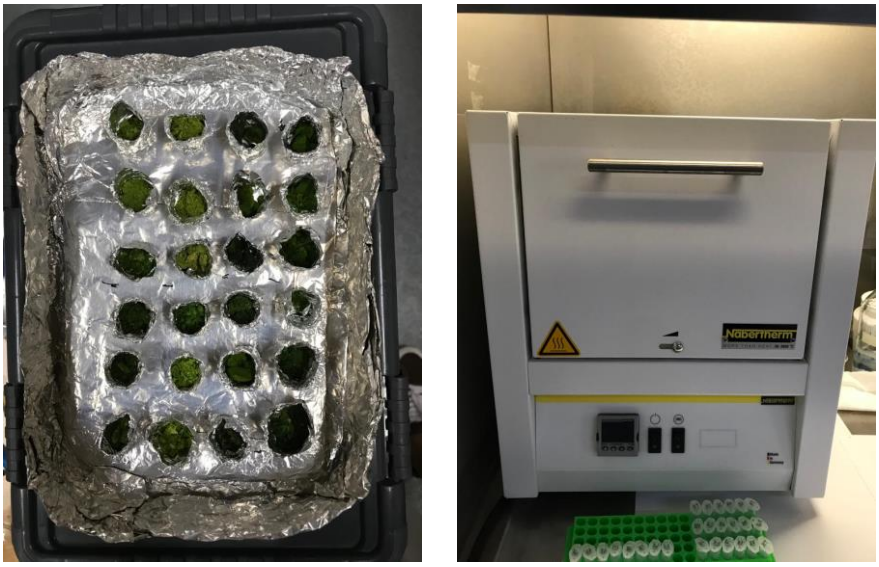


Figure 16: Images of the ash analysis method. On the left the small aluminium cups with biomass before being placed in the muffle. On the right the muffle used.

2.6.5. Carbohydrates content

The carbohydrate content is determined by difference, considering the percentage of total lipids, protein, and ash, as represented by the following formula:

$$\%CC = 100 - (\%crude\ fat + \%crude\ protein + \%ash)$$

2.7. Statistical analyses

The statistical analyses were performed using R software (version 4.3.1) through RStudio (version 2021.09.0) and the graphs were prepared with the software tool Excel (version 16.0, 2019). All the experimental results were considered at a 95 % confidence level, ($p< 0.05$). For each test, the mean and standard deviation of the triplicates were determined. Throughout the text, different letters were used to highlight significant differences. Data were compared using one-way ANOVA, followed by Tukey's multiple comparison tests.

3. Results and discussion

3.1. Medium formulation

In the preliminary analysis of the available waste, seven different wastes were dissolved in water in three different concentrations 1, 15 and 30 g/L. The pH and salinity were determined (Table 2), as well as the moisture content of the waste in dry powder form were also evaluated (Table 3).

According to the pH register (Table 2), there were no significant differences between the higher concentrations (15 g/L and 30 g/L). On the other hand, when comparing these two concentrations with 1 g/L, there is a decrease in pH, with 1 g/L always representing a higher pH. Concerning salinity (Table 2), it increases with the concentration of waste in the solution, so at 1 g/L of waste salt concentration was approximately 0 g/L.

Table 2: Results obtained for the pH and salinity of the waste for the 3 concentrations under study.

Waste	**Concentration (g/L)**	**pH ± 0.1**	**Salinity± 1 (g/L)**
7	1	6.3	0
	15	5.3	3
	30	5.2	6
10	1	5.9	0
	15	5.1	4
	30	4.9	5
12	1	5.9	0
	15	4.0	1
	30	3.9	4
13	1	5.1	0
	15	4.6	5
	30	4.3	8
15	1	6.2	0
	15	5.3	2
	30	4.8±	4
16	1	6.8	0

	15	6.5	1
	30	6.4	1
17	1	5.6	0
	15	4.3	2
	30	4.2	3

Considering the percentage of moisture content of the waste (Table 3), **Waste 16** shows significant differences compared to the other wastes (*p<0.05*). Higher humidity suggests low stability of waste 16 due to susceptibility to contamination and degradation.

Table 3: Results obtained for the percentage of moisture on different wastes.

	Moisture content (%)
Waste 7	4.758[b]
Waste 10	2.471[b]
Waste 12	3.408[b]
Waste 13	4.769[b]
Waste 15	5.234[b]
Waste 16	30.3
Waste 17	4.374[b]

3.2 Nutritional analysis of waste

The concentrations of iron (Fe), magnesium (Mg) and nitrates (NO_3^-) were analyzed in the seven wastes but were found to be below the detection limit of the methods used ($8.95* 10^{-7}$ mM, 0.025 mM and 0.01 mM, respectively), and therefore their concentration was considered negligible in the wastes.

Concerning the concentration of urea (mM) in the different wastes (Figure 17), it is noticeable that as we increase the concentration of waste in the solution, the higher

becomes the concentration of urea. Given the results obtained, we can also verify that **Waste 16** shows lower concentrations of urea for the three concentrations under study. Taking into account the culture media described in the bibliography and widely used, we can ascertain that, except for **Waste 16**, at 15 g/L of waste, there would already be enough available nitrogenous compounds, as commonly used in a nutrient medium (5 mM) (Andersen, 2005).

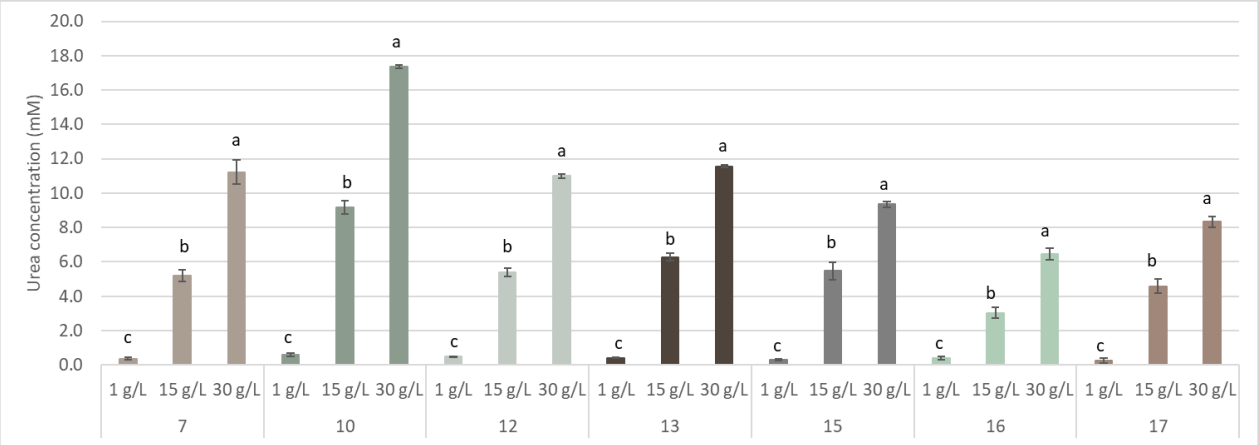


Figure 17: Urea concentration (mM) of all residues at the 3 concentrations under study. The results represent the mean of 3 replicates ± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments, compared among conditions for each residue ($p<0.05$).

To understand if the solutions become saturated, given the concentration of urea available in the solution, its concentration per gram of waste was evaluated (Figure 18). From Figure 18, we can infer that there were no significant differences between the concentrations of urea per gram of waste at waste concentrations of 15 g/L and 30 g/L, except for **Waste 16** ($p<0.05$). Compared to the other wastes, **Waste 16** showed a higher decrease between the waste concentration at 1 g/L and the two higher concentrations.

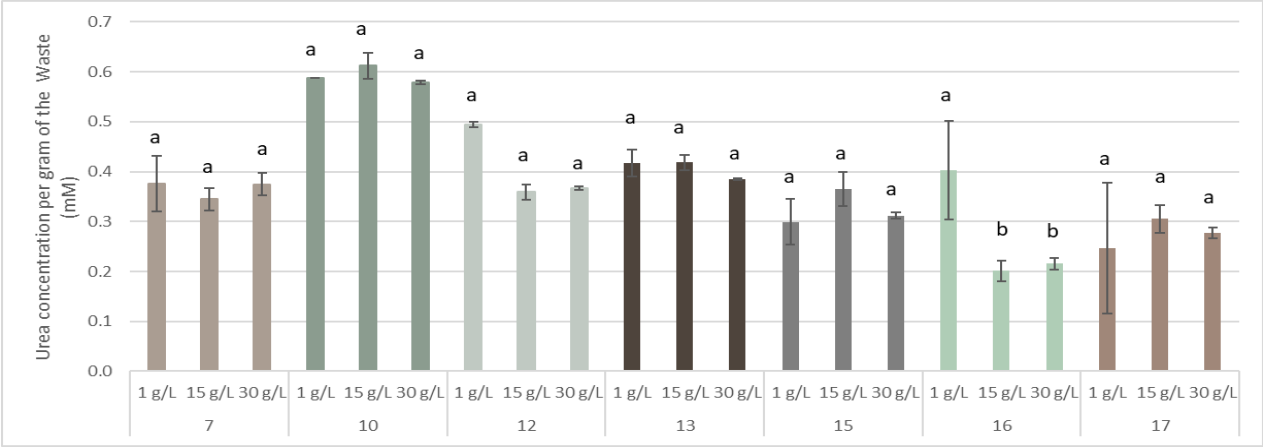


Figure 18: Urea concentration per gram of waste (mM/ g of Waste) of all wastes at the 3 concentrations under study. The results represent the mean of 3 replicates ± standard deviation. Letters "a" and "b" demonstrate significant differences in the different treatments of each residue (*p<0.05*).

By analysing the ammonia concentration (Figure 19), only **Waste 16** shows detectable concentrations, taking into account the kit used, i.e. the remaining wastes most likely have only residual concentrations, below the detection limit (0.025 mM). The concentration of ammonia in **Waste 16** may be associated with the degradation of urea that would have been present initially since this was the waste that showed the lowest values of urea (Figure 17) and the highest percentage of moisture (Table 2).

A k**B**

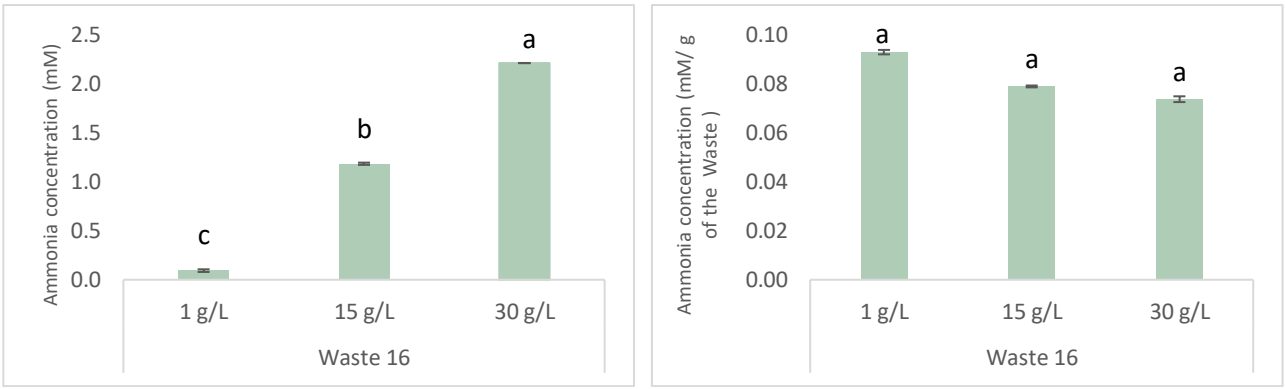


Figure 19: Results obtained of ammonia concentration. *(*A) Ammonia concentration (mM) in Waste 16 at the different concentrations evaluated (B) Ammonia concentration per gram of waste (mM/ g of Waste) in Waste 16 at the different concentrations analyzed. The results represent the mean of 3 replicates ± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments (*p< 0.05*).

These ammonia results and the fact that no nitrates could be detected suggest that urea is the main source of nitrogen when using this waste as a culture medium for microalgae.

Despite this, the concentration of ammonia in Figure 19A is shown to be increasing as the concentration of waste increases in solution. Evaluating the concentration of ammonia per gram of Waste (Figure 19B), there are no significant differences in different concentrations of **Waste 16**.

The results obtained for phosphate concentration showed that it was not possible to detect phosphate at the 1 g/L waste concentration, which is below the detection limit of the kit used (0.01 mM). Figure 20 once again highlights what has been observed concerning the compounds previously analyzed, revealing that a higher concentration of waste in solution is associated with a higher concentration of available phosphate. According to the commonly used culture media found in the bibliography, at 15 g/L of waste in solution, there is enough phosphate (0.05 mM) (Andersen, 2005), except for **Waste 16,** Figure 20.

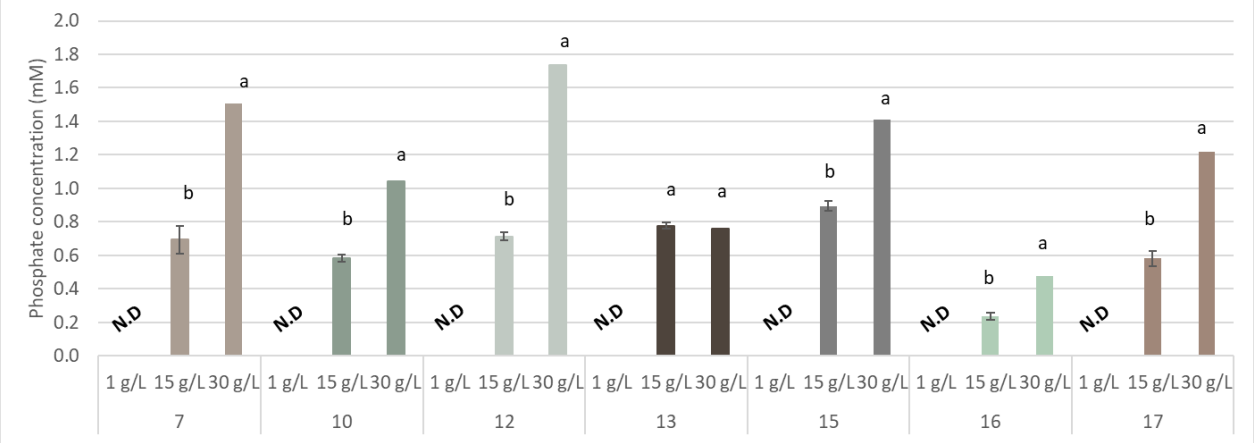


Figure 20: Phosphate concentration (mM) of all residues at different concentrations . The results represent the mean of 3 replicates ± standard deviation and N.D. corresponds to the concentrations that were under the detection limit. Letters "a", and "b" demonstrate significant differences in the different treatments ($p< 0.05$).

Analysing the phosphate concentration per gram of waste (Figure 21), we can infer that significant differences were only observed between the concentrations 15 g/L and 30 g/L in **Wastes 13 and 15**, resulting in a decrease in phosphate concentration ($p< 0.05$). On the other hand, in the remaining Wastes, no significant differences were observed in the different concentrations in which it was detected ($p< 0.05$). This suggests that 30 g/L is

a high concentration to obtain the desired phosphate concentration. The same was true of the urea concentration, so 15 g/L is the optimum concentration to apply.

It should also be considered that the kit only allows measuring orthophosphate, and thus not necessarily all phosphate.

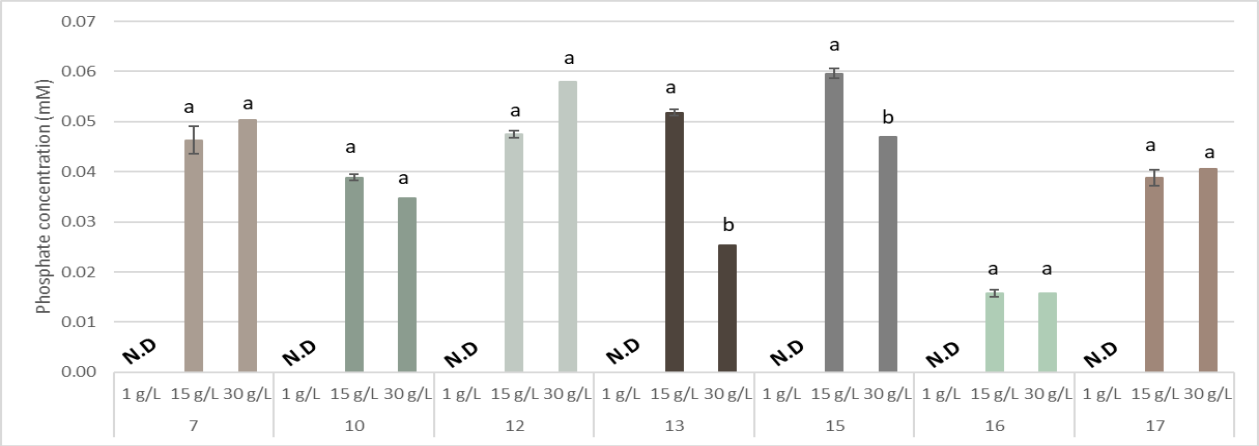


Figure 21: Phosphate concentration per gram of residue (mM/ g of Waste) of all wastes tested. The results represent the mean of 3 replicates ± standard deviation. Letters "a" and "b" demonstrate significant differences in the different treatments, among each waste ($p< 0.05$).

To complete this preliminary analysis of available Wastes, to proceed to *Chlorella vulgaris* growth studies, the nitrogen/phosphate ratio in the different Wastes was evaluated (Figure 22). Taking into account, the ratio that is commonly used in culture media for microalgae (N/P=18) (Andersen, 2005; Molazadeh et al., 2019), only Wastes 10, 13 (only at 30 g/L) and 16 reach this value. In the study carried out by Mao, the N/P=18 ratio proved to be the optimum ratio for growing *Chlorella*, with higher chlorophyll and lipid values. The study also emphasizes that ratios of 13, 15 or 22, despite having lower dry weights, show better results in terms of protein concentration. (Mao et al., 2023).

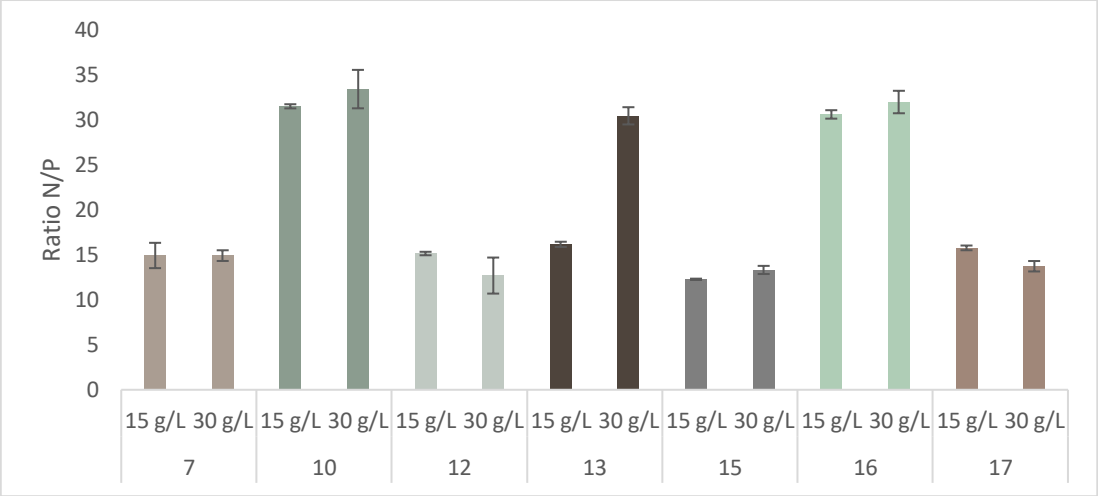


Figure 22: Nitrogen/phosphate ratio of all Wastes at the concentrations tested in the conditions where phosphate was detectable. The results represent the mean of 3 replicates ± standard deviation.

3.3 *C. vulgaris* screening assay

C. vulgaris was grown in **Wastes 7, 10, 12, 13, 15, and 17** dissolved at 15 g/ L, since this demonstrated the sufficient nutrient concentration (**3.1 Medium formulation**). **Waste 16** in this test was excluded from the rest of the studies due to its low stability.

The growth curves obtained from this batch trial (Figure 23), suggest the wastes are quite comparable with each other, with *C. vulgaris* reaching a final dry weight close to 1 *g/L*, prior to any optimization.

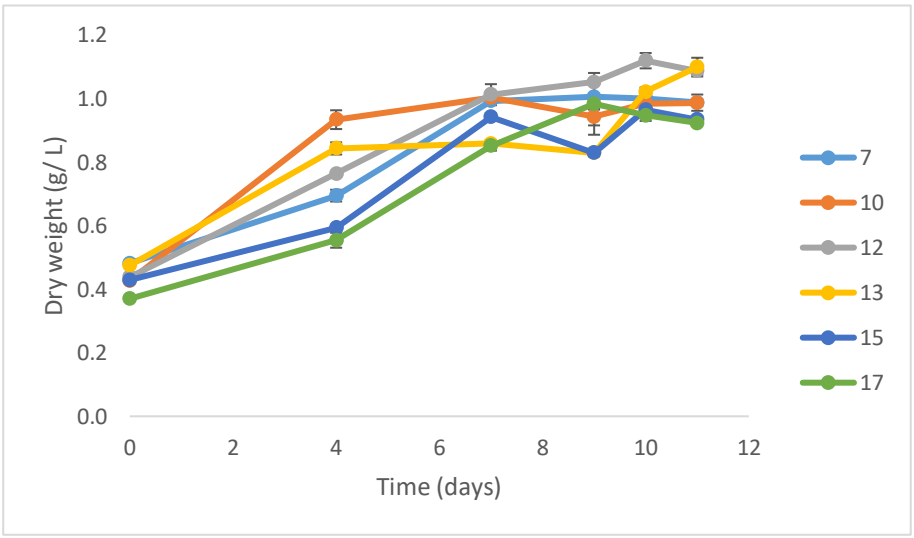


Figure 23 *Chlorella vulgaris* growth curves, on the different wastes at 15 g/ L concentration in the inoculum room. Culture was grown at an initial dry weight of 0.4 g/L on 0.5 L reactors at 20 ºC. The results represent the mean of 3 replicates ± standard deviation.

From these growth curves there is no single waste that stands out as best performing, however other parameters such as Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (μ) and Cell density were also evaluated (Figure 24).

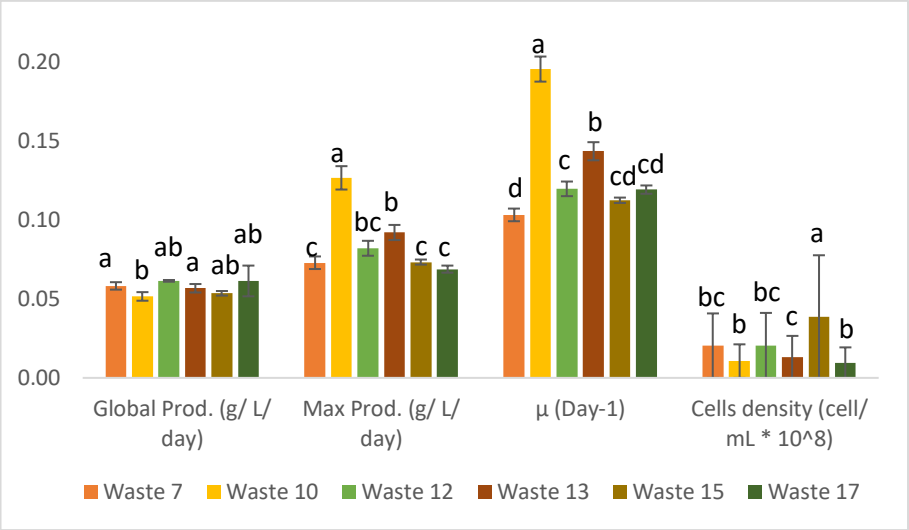


Figure 24: Results of Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (μ) and Cell density of *Chlorella vulgaris* grown in 6 different Wastes as culture media at 15 g/ L concentrations. Culture was grown at an initial dry weight of 0.4 g/L on 0.5 L reactors at 20º C. The results represent the mean of 3 biological replicates± standard deviation. Letters "a", "b" and "c" demonstrate significant differences between different treatments, among each parameter evaluated ($p< 0.05$).

The analysis of the different parameters of Figure 24 indicates that **Waste 10** is the most promising given its higher **Max Productivity** and specific growth rate (**μ**) ($p< 0.05$). Still, when evaluating through absorbance, there might be interference of small particles in suspension present in the supernatant and for this reason cell density was also evaluated **Waste 15** also showed promising results compared to the other Wastes, considering Cell density ($p< 0.05$), which also highlights its potential.

Considering the nutrients present in the different Wastes (urea and phosphate), their consumption throughout the trial was also evaluated.

The results of the consumption of nitrogen and phosphate suggest they were consumed during the trial. The curves of Figure 25 enhance that the urea and phosphate were almost entirely consumed by *C. vulgaris* or by the microorganisms present in Waste that were not previously autoclaved.

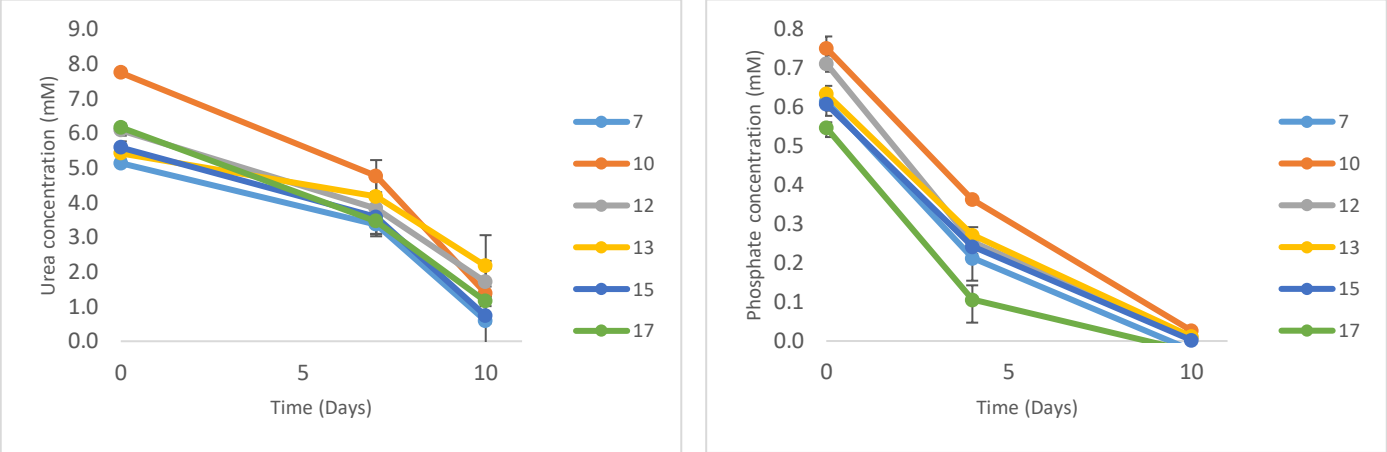


Figure 25: Consumption of Nitrogen (urea) and Phosphate (mM) during the 11 days of the trial under the tested conditions in which 6 different Waste as culture medium at 15 g/ L concentration were used. Culture was grown at an initial dry weight of 0.4 g/L on 0.5.L reactors at 20º C. The results represent the mean of 3 replicate ± standard deviation.

Taking into account the results observed in this assay, **Wastes 10 and 15** proved to be the most promising ones. Although **Waste 15** proved to be a promising waste according to results less affected by interferences. Comparing both wastes composition, **Waste 10** shows to be more promising because it has one more food category (bread), while on preparation of **Waste 15** the addition of sawdust to replace bread in its constitution incurs an extra cost. The presence of sawdust or bread enhance the residue texture to prevent moisture degradation of waste, increasing its shelf life.

3.4 Micronutrients supplementation

After selecting the most promising **Waste 10**, the next step was to optimize the growth of the alga in the waste, through nutritional supplementation. Specifically, different micronutrients' solutions were added, and growth was compared to that obtained with the standard industrial medium.

Comparing the growth curves obtained in the next trial, there is a great discrepancy in the growth observed in the treatments in which **Waste 10** was used, compared to the results observed in the control (CTL). Specifically, the maximum concentration reached using Waste 10 was 0.8 g/L, while *C. vulgaris* grown with the company's standard culture medium achieved 2.1 g/L (Figure 26).

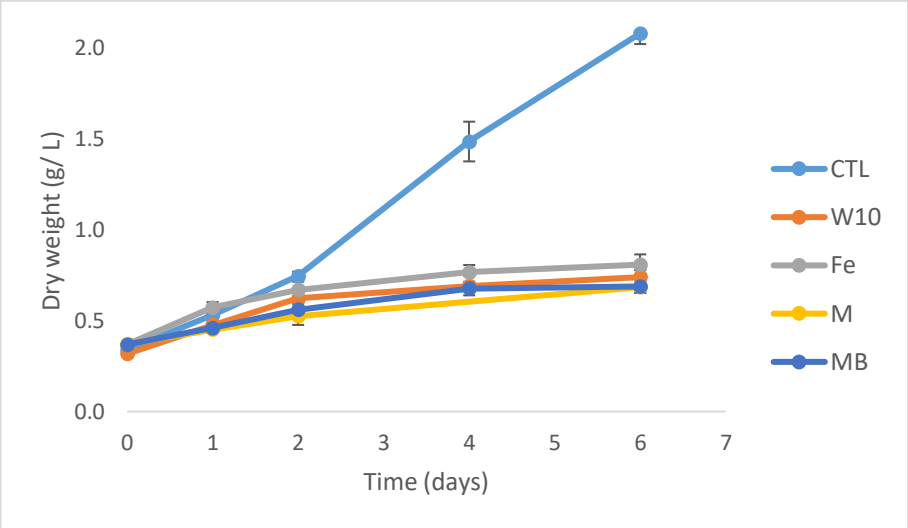


Figure 26*: Chlorella vulgaris* growth on the Waste 10 at 15 g/ L in the inoculum room, with supplementation of four solutions*:* Iron solution (Fe); Solution with micronutrients for inorganic growth (M); Solution with micronutrients for organic growth (MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. CTL represent the control (with inorganic culture medium) and W10 the use of the Waste 10 without supplementation. The results represent the mean of 3 replicate ± standard deviation.

Since there were no significant differences in the treatments where **Waste 10** was used (whether or not it was supplemented with micronutrients), this suggests that growth impairment was not due to lack/excess of micronutrients (Figure 27). Still, growth in **Waste 10** was considered not to be competitive with the control medium.

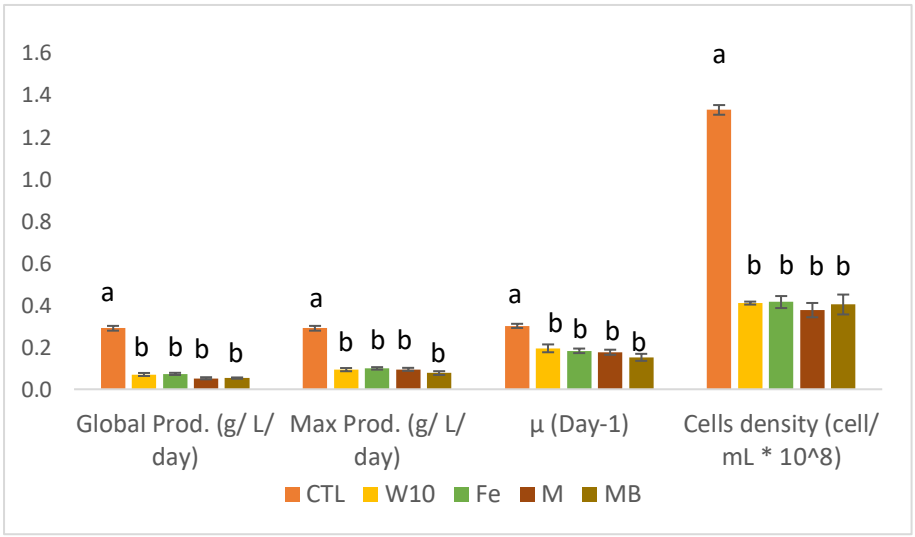


Figure 27: Results of Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (µ) and Cell density under the tested conditions, in particular, using industrial medium (CTL), or Waste 10 supplied of four solutions: Iron solution (Fe); Solution with micronutrients for inorganic growth (M); Solution with micronutrients for organic growth (MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. CTL represent the control (with inorganic culture medium) and R10 the use of the Waste 10 without supplementation. The results represent the mean of 3 replicate ± standard deviation. Letters "a" and "b" demonstrate significant differences in the different treatments, among each parameter evaluated ($p < 0.05$).

Evaluation of the Nitrogen and Phosphate consumption throughout the trial (Figure 28) indicates they were only completely consumed in the control condition, CTL treatment, which indicates that the macronutrients present in the waste were only partially available for uptake by the alga. Furthermore, since there was no relevant microalgal growth, urea and phosphate were most likely consumed by microorganisms other than *Chlorella* (**3.3 Screening assay**).

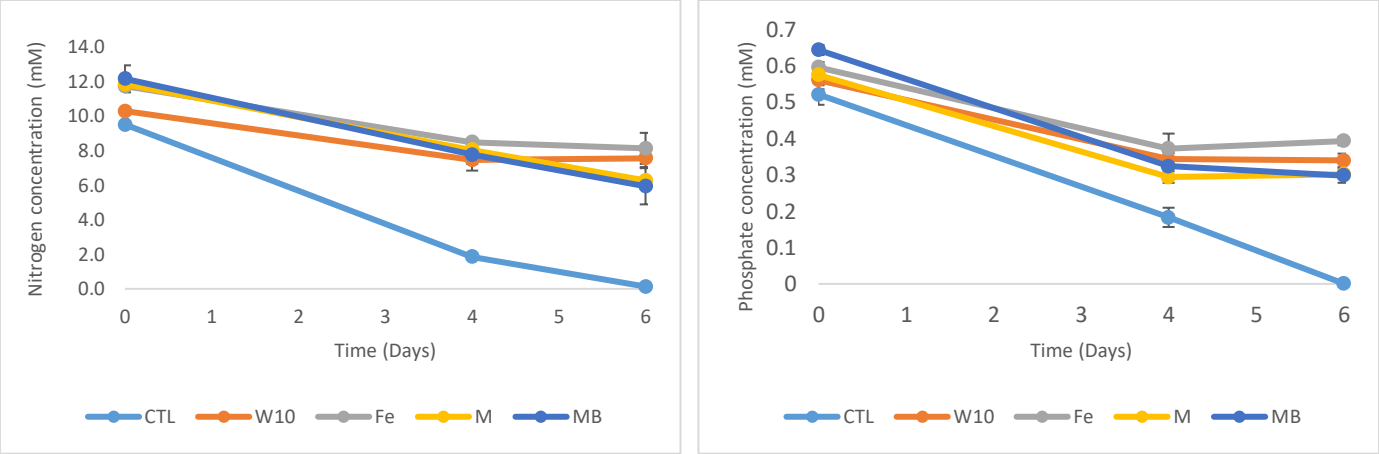


Figure 28: Consumption of Nitrogen (urea) and Phosphate (mM) during the 6 days of the trial under the tested conditions using industrial medium (CTL), or Waste 10 supplied of four solutions: Iron solution (Fe); Solution with micronutrients for inorganic growth (M); Solution with micronutrients for organic growth (MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. CTL represent the control (with inorganic culture medium) and R10 the use of the Waste 10 without supplementation. The results represent the mean of 3 replicate ± standard deviation.

3.5 Macronutrients supplementation

Next, a trial was performed, where macronutrients were supplemented (one at a time) to check whether each one was a limiting factor to *Chlorella* growth in the waste. Despite it being detected in the medium, it might not be bioavailable to the alga and therefore would require supplementation.

Figure 29, shows that using the waste with the supplementation of nitrogen, it is possible to obtain a growth profile comparable to that enabled by the culture medium of the company. Supplying phosphate, potassium, or calcium to Waste 10 is not necessary, as growth was similar to that observed with no supplementation of any nutrient (W10).

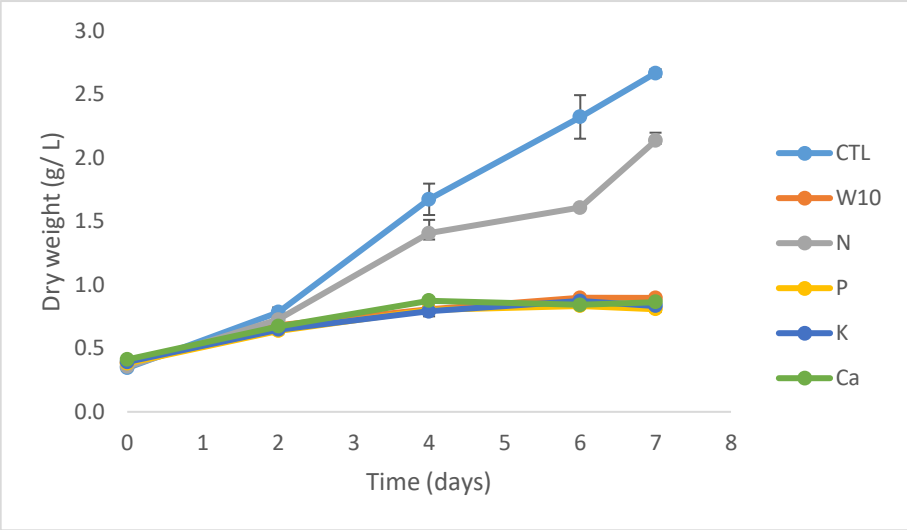


Figure 29: *Chlorella vulgaris* growth curves on the Waste 10 at 15 g/ L in the inoculum room, with supplementation of different solutions: nitrogen solution (N); phosphate solution (P); potassium solution (K); calcium solution (Ca). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. CTL represents the industrial medium as positive control and W10 represents the use of Waste 10 without supplementation. The results represent the mean of 3 replicate ± standard deviation.

By analyzing the productivity, specific growth rate and cell density, the culture medium used by the company stands out, with significant differences compared to the other treatments ($p< 0.05$). However, nitrogen supplementation showed significant differences to other treatments where **Waste 10** was used, with values significantly higher than in the other treatments ($p< 0.05$) (Figure 30).

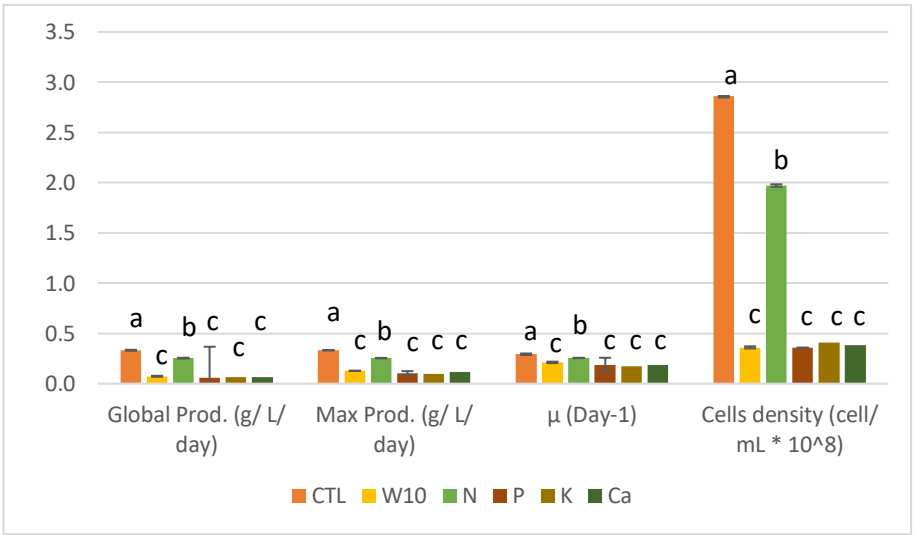


Figure 30: Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (µ) and Cell density, under the tested conditions, in particular, using industrial medium (CTL), or Waste 10 without supplementation (R10), or supplementing it with nitrates (N), phosphate (P), potassium (K), or calcium (Ca). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

This is in line with the results obtained in Ana Abreu's study, in which she tested the cultivation of *C. vulgaris* in 4 culture media with powdered waste from a dairy company. She showed that optimizing the media with supplementation led to a doubling of the results observed in terms of specific growth rate, overall yield and maximum yield (Abreu et al., 2012). These data suggest that it may also be advisable to supplement the media to obtain competitive results using the current culture medium. As expected given the study carried out by Ana Abreu, supplementation enabled the results observed in the previous trial (**3.4 Micronutrient supplementation**) and the treatment using **Waste 10** without supplementation led to double yield considering specific growth rate and both overall and maximum productivities (Abreu et al., 2012).

Evaluating consumption throughout the trial, the urea of treatments R10, N, P, K and Ca continued not to be consumed by *C. vulgaris* (Figure 31). Phosphate consumption was also slightly changed (Figure 31). With nitrogen supplementation, *C. vulgaris* demonstrates total consumption of the supplemented nitrogen (nitrate form) even though it did not consume the nitrogen prevenient from the waste (urea). Phosphate is also fully consumed as soon as the microalga consumes nitrogen.

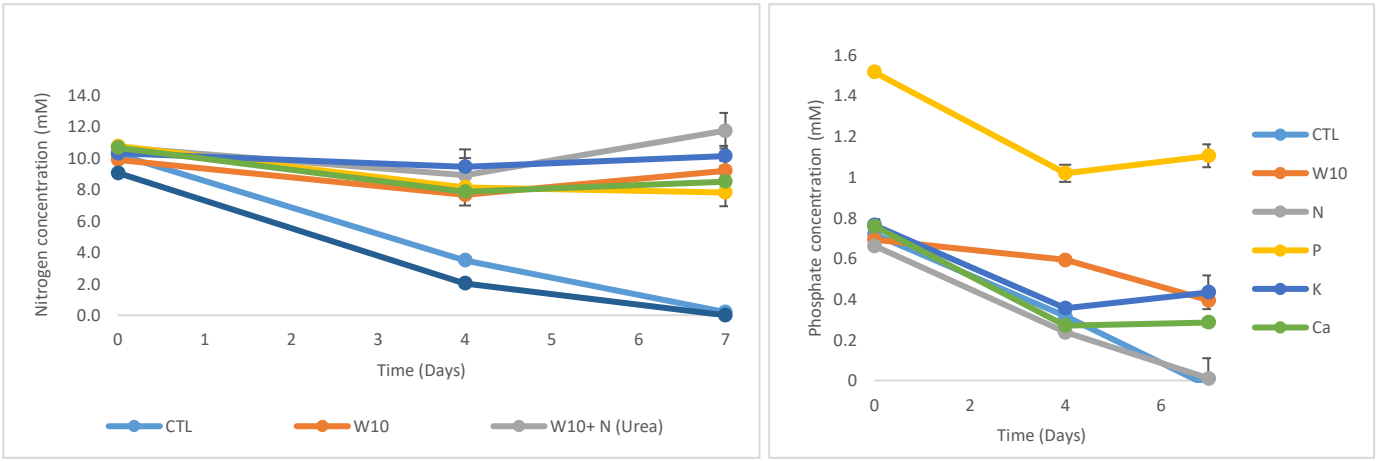


Figure 31:Consumption of Nitrogen and Phosphate (mM) during the 6 days of the trial under the tested conditions, in particular, using industrial medium (CTL), or Waste 10 without supplementation (R10), or supplementing it with nitrates (N), phosphate (P), potassium (K), or calcium (Ca). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates ± standard deviation.

These results, therefore, highlight that the use of this Waste as a culture medium is possible with the supplementation of nitrates as a nitrogen source since the present nitrogen in the form of urea most likely is not bioavailable. The results observed in this test show that it is far from the growth that has already been observed using the medium already adopted by the company, which has already been optimized. The study carried

out on *Chlorella sp.* by Vidya shows that the use of two culture media based on industrial waste results in lower growth than when using a standard, control culture medium. (Vidya et al., 2021). This suggest it will be necessary to optimize the medium under study in order to compete with those already on the market.

3.6 Growth optimization with supplementation of nitrogen and micronutrients

Next, a trial was designed where **Waste 10** was used, and in which nitrogen was supplemented in addition to each of the different micronutrient solutions, in an effort to optimize the growth.

The growth curves obtained from this trial (Figure 32), suggest it is possible to achieve productivities like those of the control medium (CTL) using the supplemented waste. These results demonstrate that **Waste 10** may be used instead of the industrial medium, which is also supplemented with iron and micronutrients, to cultivate *C. vulgaris*. The final dry weight was approximately 2.8 g/L, for the CTL, W10+ N+ Fe, and W10+ N+ MB conditions. In the treatment without iron or micronutrients supplementation, growth was lower compared to the CTL and similar to the treatment supplied with micronutrient solution for inorganic growth (M), suggesting the addition of solution M does not present the micronutrients that the culture is lacking.

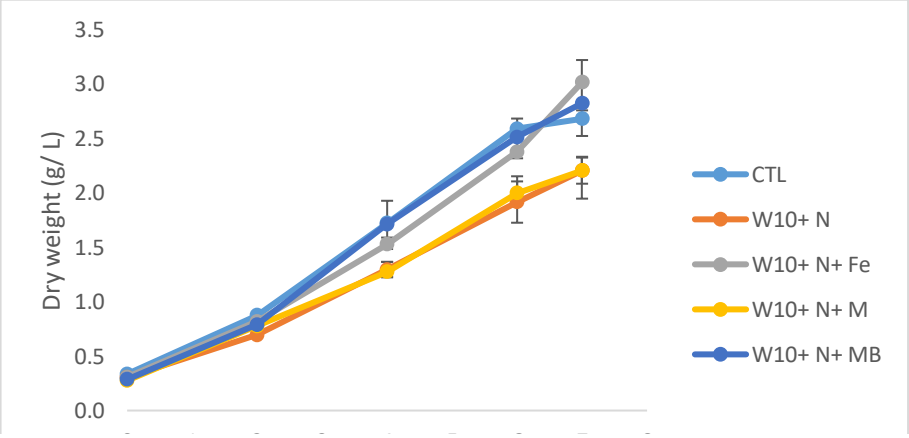


Figure 32: *Chlorella vulgaris* growth on the Waste 10 at 15 g/ L in the inoculum room, with supplementation of nitrogen and different solutions of micronutrients: Iron solution (Fe); Solution with micronutrients for inorganic growth (M); Solution with micronutrients for organic growth (MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. CTL represent the control industrial medium and N represents the use of the Waste 10 with nitrates supplementation only. The results represent the mean of 3 replicates± standard deviation.

The results of maximum productivity (Figure 33) were significantly higher in the control (CTL), the supplementation of iron (W10+ N+ Fe) and the supplementation of micronutrients for organic growth (W10+ N+ MB) compared with the treatments with no supplementation other than nitrogen (N) and the supplementation of micronutrients for an inorganic growth (W10+ N+ M) ($p< 0.05$). These results indicate that the use of iron or other micronutrients for organic growth supplementation would be beneficial to achieve the maximum productivity values observed on control (CTL). However, given the optical interference of the residue, which is inevitable in the absorbance readings, the results concerning cell density are considered the most reliable. Analysis of this graph (Figure 33) suggests that only the condition in which both nitrogen and iron were supplemented reached the same growth as the control industrial medium, showing no significant differences ($p\geq 0.05$).

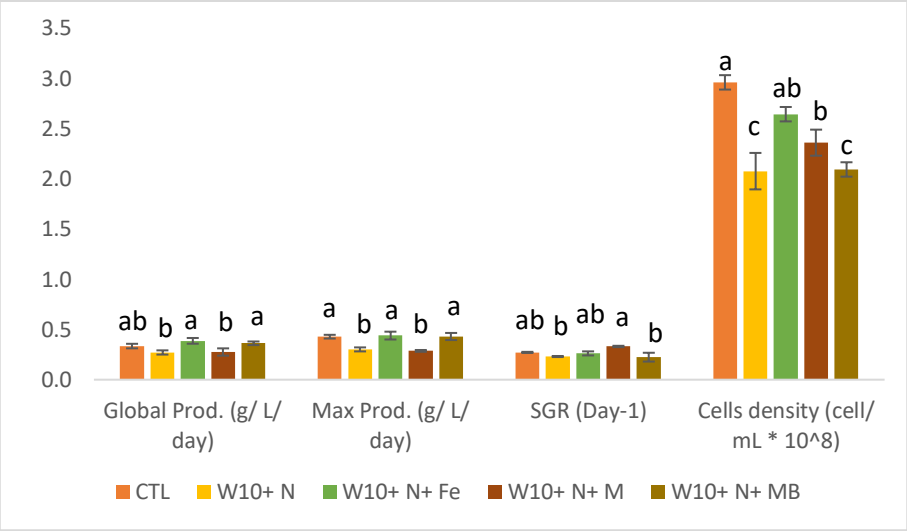


Figure 33: Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (μ) and Cell density, under the tested conditions. CTL represents the industrial medium as a positive control, and the others represent Waste 10 supplemented with nitrates (N) or nitrates plus the different micronutrient solutions (Fe, M, and MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

As noted earlier the urea present in the residue was not consumed while the supplemented nitrates were completely consumed (Figure 34). Phosphate in **Waste 10** was also consumed in all treatments (Figure 35). The treatments where waste was used showed negative nitrate concentrations at the end of the test. As negative concentrations

are not possible, the data depicted in Figure 35 most likely represent the interference of the color of the waste in the reading on the spectrophotometer, still phosphate seems to be completely absent in the final stages of the assay.

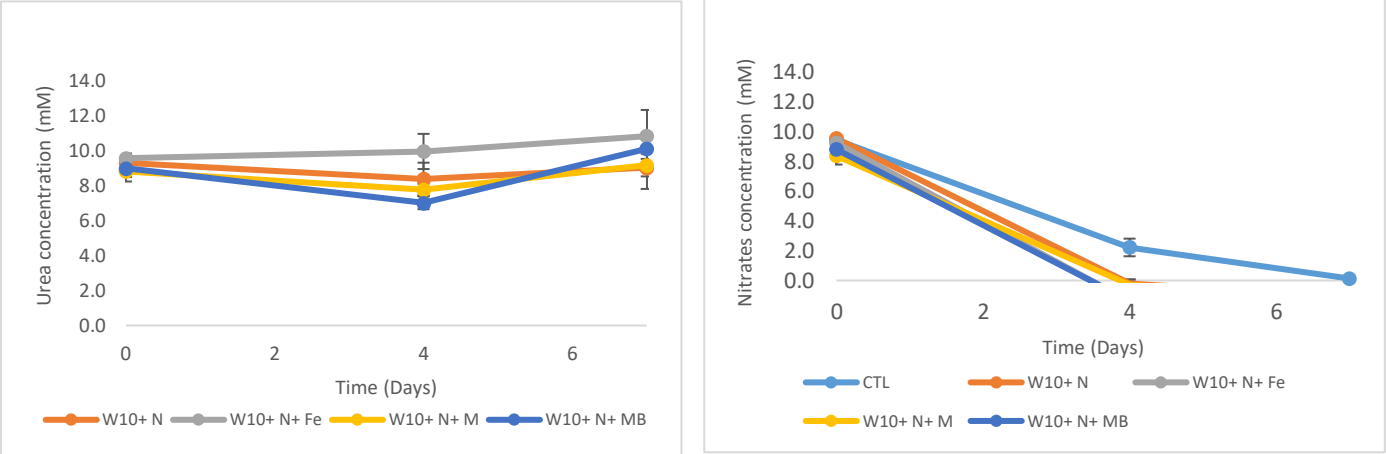


Figure 34: Consumption of nitrogen (mM) of different sources: urea (left) and nitrates (right) during the 6 days of the trial. *CTL represents the industrial medium as a positive control, and the others represent Waste 10 supplemented with nitrates (N) or nitrates plus the different micronutrient solutions (Fe, M, and MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation.*

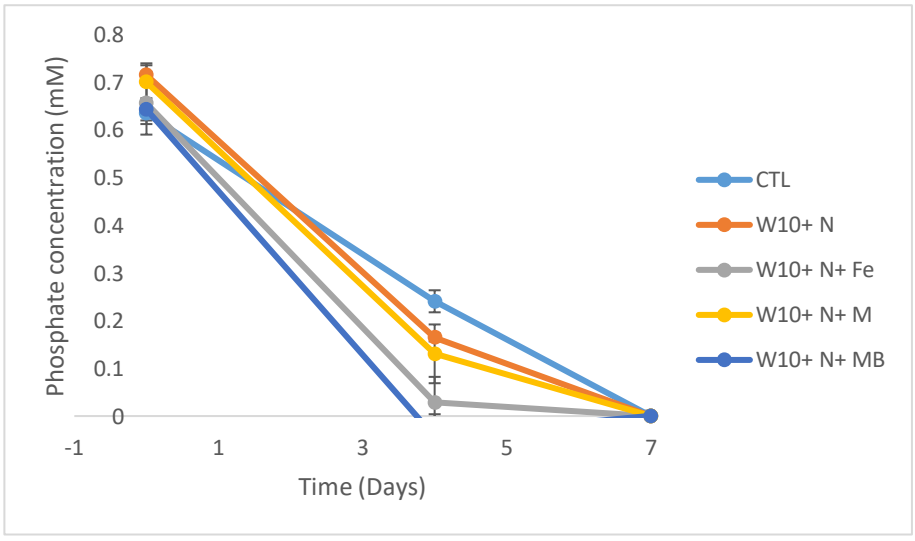


Figure 35: Consumption of phosphate (mM) during the 7 days of the trial. CTL represents the industrial medium as a positive control, and the others represent Waste 10 supplemented with nitrates (N) or nitrates plus the different micronutrient solutions (Fe, M, and MB). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation.

3.7 Laboratorial validation assays

To validate the results obtained on previous trials, a trial with the supplementation of iron and nitrogen treatment (Fe) was performed to compare the supplemented **Waste 10**

medium to other existing formulations, namely inorganic (Ino) and organic (Org) culture media that are commonly used at Allmicroalgae. The treatment without any supplementation (W10) was compared, as a baseline, plus treatment with the supplementation of micronutrients of inorganic cultivation (MF), also containing iron.

Figure 36 shows growth curves obtained from this batch growth trial and the curves of Ino, Fe and MF demonstrate no significant differences ($p \geq 0.05$) from each other. As it was expected, the use of Waste with supplementation and the inorganic culture medium stood out from the use of organic culture medium (Org) and the non-supplementation of **Waste 10**.

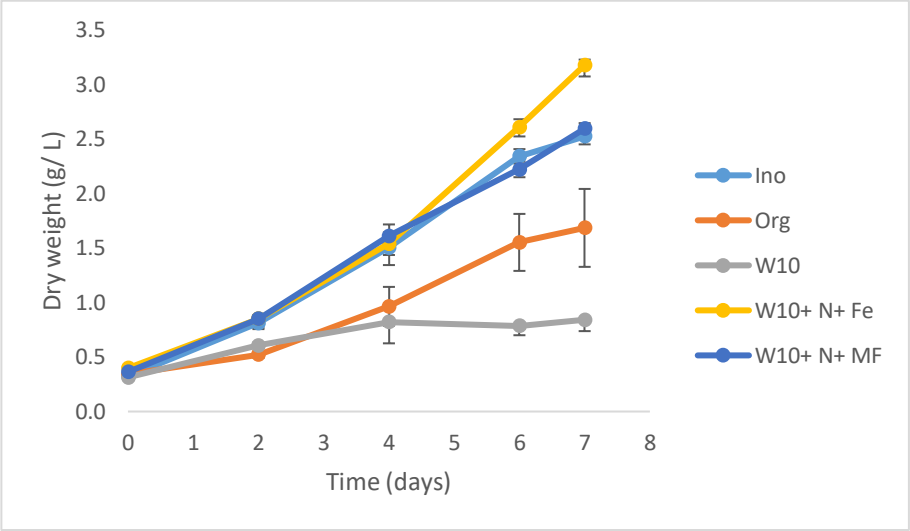


Figure 36: *Chlorella vulgaris* growth on Waste 10 at 15 g/ L concentration in the inoculum room. Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and MF, supplied an iron solution and micronutrients of an inorganic growth solution. Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation.

The global productivity (Figure 37) of Waste 10 supplemented with N+ Fe was significantly higher than all the others and similar to the one supplemented with N+MF ($p < 0.05$) . This last condition had a productivity equal to the inorganic control (Ino) ($p \geq 0.05$) and significantly higher than the organic control (Org) ($p < 0.05$). On cell density there were no significant differences between the treatment with the residue (W10) and the inorganic control (Ino) ($p \geq 0.05$), and they show higher values than those obtained with the organic medium (Org), with significant differences ($p < 0.05$).

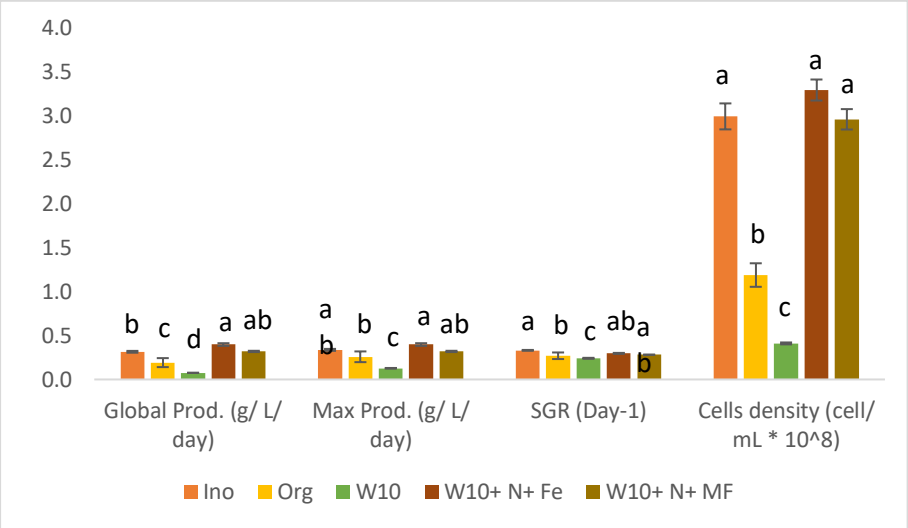


Figure 37: *Chlorella vulgaris* growth on Waste 10 at 15 g/ L concentration in the inoculum room. Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (μ) and Cell density, under the tested conditions. Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (W+ N) plus the different micronutrient solutions: Fe (iron only) and MF (an inorganic solution containing both iron and micronutrients solution). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

These results indicate that the use of micronutrients for organic growth supplementation is not necessary, whereby the addition of micronutrients would be an extra cost, but not necessary to improve the growth.

Based on the data depicted in Figure 38, only the supplemented nitrates were consumed. The phosphate present in the Ino culture medium of the company and on supplemented Waste 10 was also consumed throughout the trial (Figure 39).

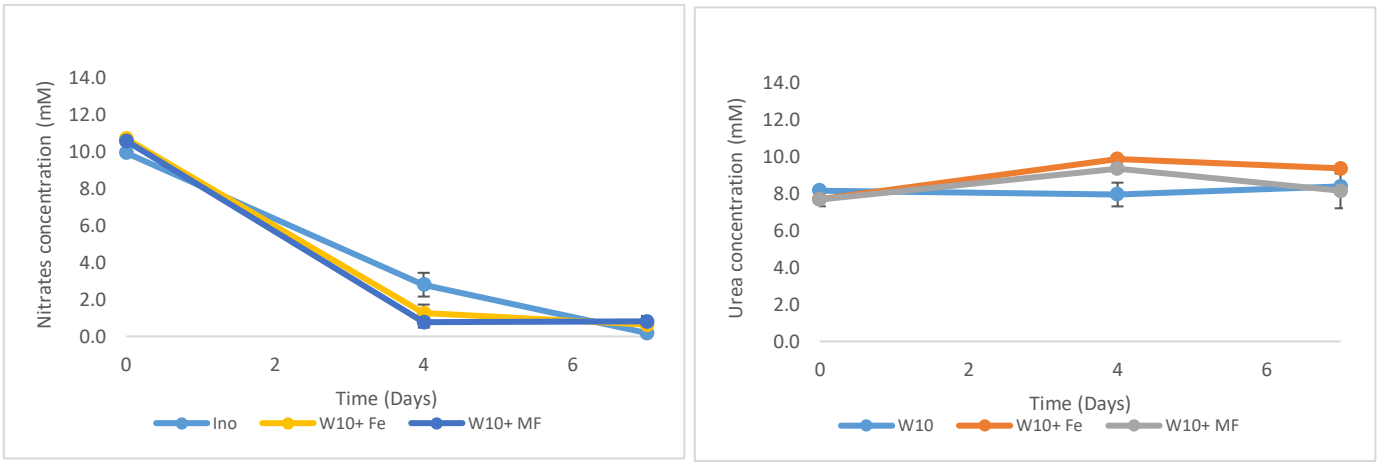


Figure 38: *Chlorella vulgaris* consumption of nitrates (left) and urea (right) on the 7 days of trial. Ino and Org represent the industrial medium as a positive control inorganic and organic culture media respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe (iron only) and MF (iron and micronutrients solution). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation.

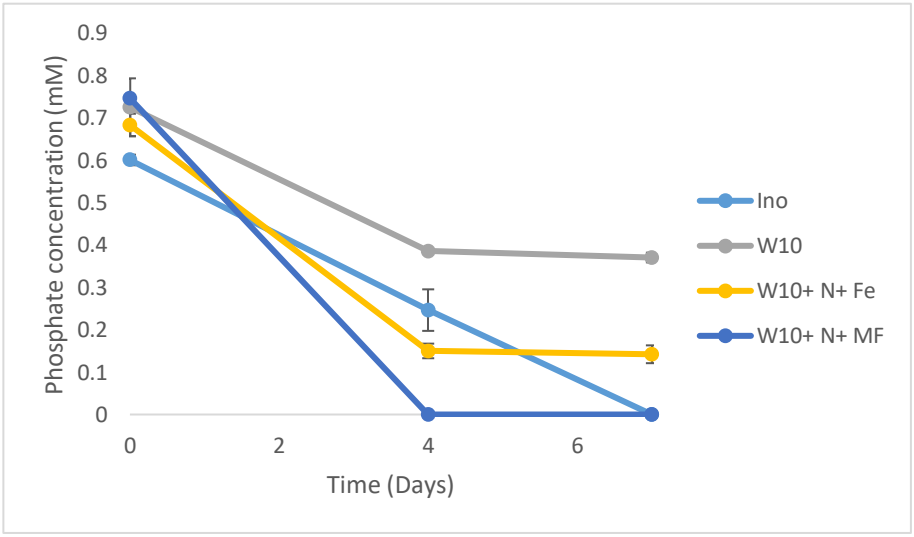


Figure 39: *Chlorella vulgaris* consumption of phosphate on the 7 days of trial. Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and MF, supplied an iron solution and micronutrients of an inorganic growth solution. Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation.

3.8 Outdoor validation

Considering the results obtained in the inoculum room, the next step was to assess growth of *C. vulgaris* in outdoors bubble columns. Autotrophic microalgae are cultivated industrially in similar conditions, exposed to ambient light and temperature, thus this assay is representative of the industrial settings/conditions. On this assay, the supplementation of iron and nitrogen treatment (N+ Fe) on **Waste 10** was performed to compare the growth, according to environmental conditions, on other existing formulations, namely inorganic (Ino) and organic (Org) culture media that are commonly used at Allmicroalgae and that are already optimized.

The growth curves obtained from this trial show a decrease in the final dry weight compared to that obtained in **3.7**, not exceeding 2 g/L (Figure 40). This result may have been influenced by the photoperiod or atmospheric conditions. However, the growth curves of the inorganic medium and the waste are similar to one another.

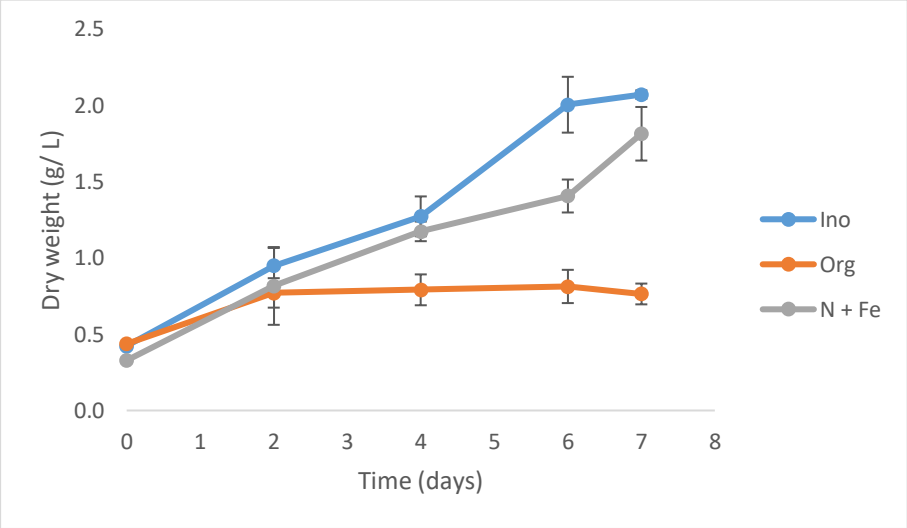


Figure 40: *Chlorella vulgaris* growth on Waste 10 at 15 g/ L concentration outdoors bubble column reactors. Ino and Org represent the industrial medium as a positive control inorganic and organic culture media respectively, and the other represents Waste 10 supplemented with nitrates plus an iron solution (N+ Fe). The results represent the mean of 3 replicates± standard deviation.

According to Figure 41, the results obtained using the inorganic medium (Ino) and the **Waste 10** (N+ Fe) showed higher values in all the evaluated parameters comparing to those obtained using the organic medium (Org). The use of the inorganic medium or supplemented Waste 10 did not show significant differences ($p \geq 0.05$), so the use of supplemented Waste 10 as a culture medium could be an alternative.

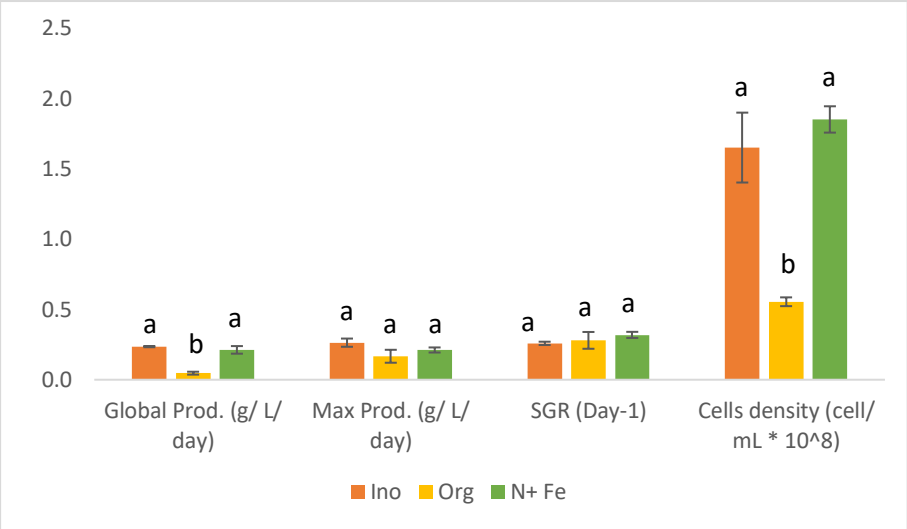


Figure 41: *Chlorella vulgaris* growth on Waste 10 at 15 g/ L concentration outdoors bubble column reactors. Global Productivity (Global Prod.), Max Productivity (Max Prod.), Specific growth rate (μ) and Cell density, under the tested conditions. Ino and Org represent the industrial medium as a positive control inorganic and organic culture media respectively, and the other represents Waste 10 supplemented with nitrates plus an iron solution (N+ Fe). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

On the other hand, the results obtained with the company's organic medium were lower than expected given what was previously observed in the inoculum room. However, this result may have been influenced by the photoperiod or atmospheric conditions.

Based on the consumption of nutrients depicted in Figure 42, as observed before, only the supplemented nitrates were consumed. The phosphate present in the Ino culture media of the company and on supplemented **Waste 10** was also consumed throughout the trial (Figure 42).

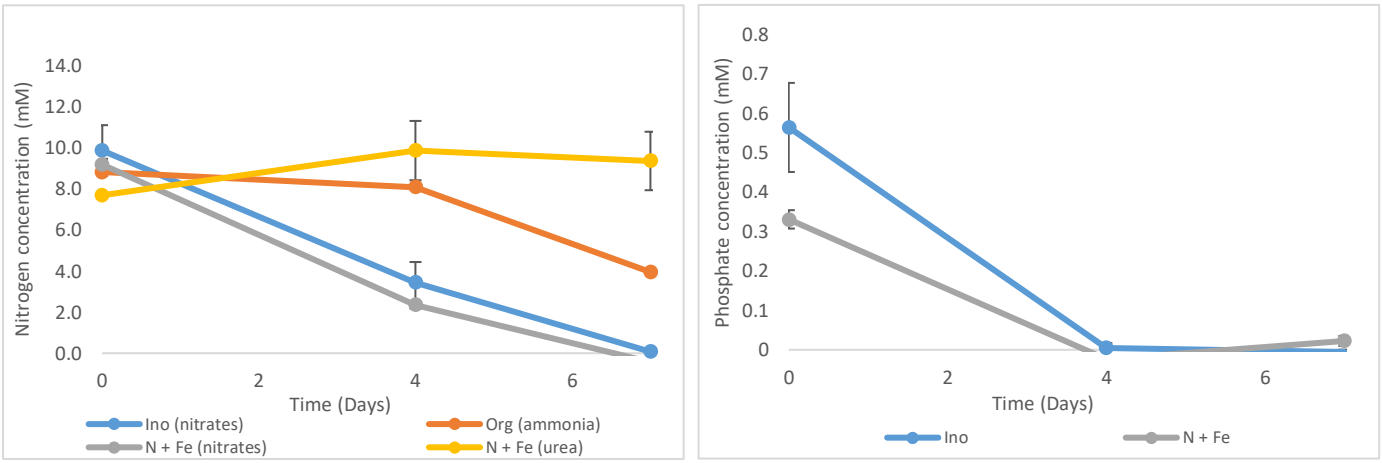


Figure 42: *Chlorella vulgaris* consumption of nitrogen (left) and phosphate (right) on the 7 days of trial. Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 supplemented with nitrates (N) plus an iron solution (N+ Fe). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation.

These results obtained with the use of **Waste 10** as a culture medium demonstrate it is possible to grow *C. vulgaris* using the waste as medium supplemented with nitrogen and iron maintaining similar productivities to those of the culture grown with inorganic medium. One the other hand, it would be pertinent to optimize it further so that nitrogen supplementation would not be necessary. Furthermore, because not all nitrogen is consumed, not all is removed from the waste which will still be discarded at the end of a microalgae production cycle, thus the risk of environmental eutrophication remains. However, it was possible to assimilate all the phosphorus present in the waste (bioremediation).

The results show that the nitrogen source is not available to *C. vulgaris* even though it has been shown capable to assimilate nitrogen from urea (Kong et al., 2011; Pozzobon et al., 2021). Furthermore, this might not be true for other species, which may be able to convert/modify and uptake the (presumably complex) urea. Some studies have shown that pre-treatment of waste leads to better results by increasing the bioavailability of the nutrients present for its use as a culture medium. For instance, the use of hydrolytic enzymes has been shown to increase bioavailability (Leung et al., 2012; Zhang et al., 2013).

3.9 Biochemical analyses

3.9.1 Protein content

Either in the indoor and outdoor assays (Figure 43), the treatment in which the company's organic medium was used stood out significantly from the other treatments ($p< 0.05$). Specifically, in the inoculum room, there were significant differences in protein content between the supplemented alternative formulation (W10+N+ Fe and W10+ N+ Fe+ M) compared to the inorganic medium control ($p< 0.05$). The treatments with supplemented waste (W10+N+ Fe and W10+ N+ Fe+ M) showed a significantly lower percentage of protein than biomass obtained with the company's inorganic medium (Ino). As expected, the results obtained in the non-supplemented medium (W10) show a lower percentage of proteins.

However, this was not the case when the assay was repeated outdoors. The culture grown with the waste supplemented with nitrogen and iron (W10+ N+ Fe) showed similar protein content as the culture grown with company's inorganic medium ($p\geq 0.05$).

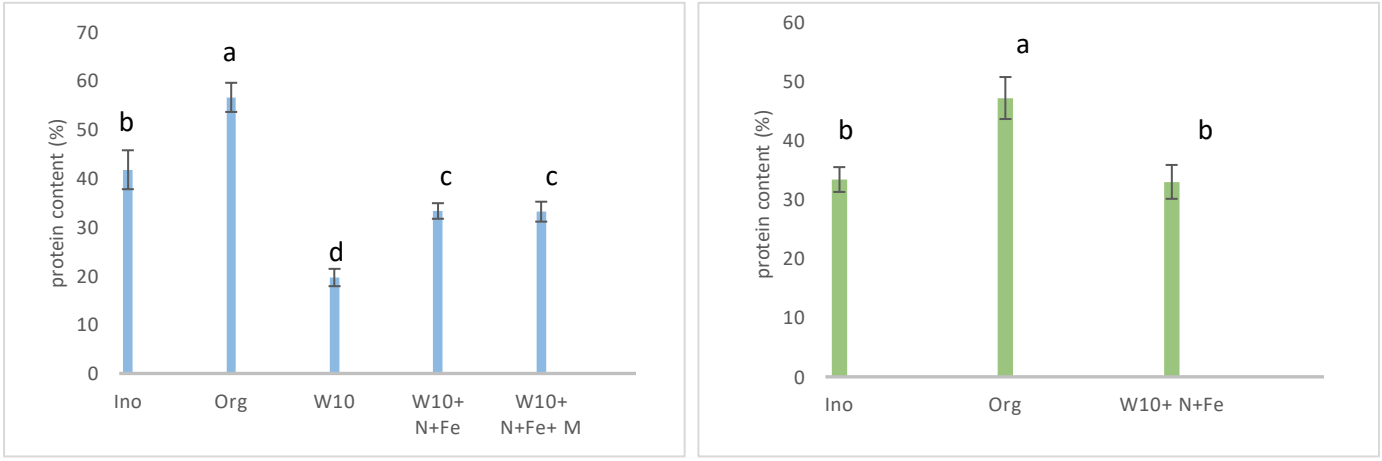


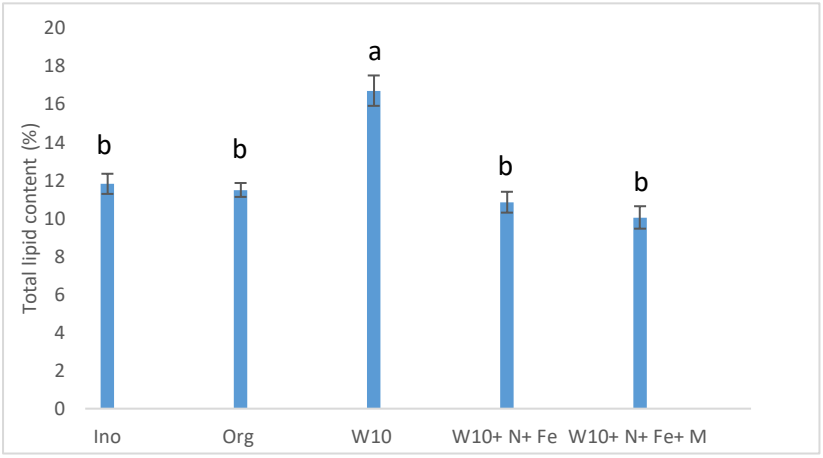
Figure 43: Protein content (%) of Chlorella biomass produced in inoculum room (3.7) (left) and on bubble columns (3.8)(right). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and Fe+ M, supplied an iron solution and micronutrients of an inorganic growth solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

The study carried out by Ana Abreu shows a protein content above 50% for *C. vulgaris* grown on oat and cheese industrial residues (Abreu et al., 2012). These were higher than those found in the present study, however, this discrepancy in values could be related to the use of different industrial wastes.

3.9.2 Lipid content

Considering both tests, significant differences in lipid content were only observed on the trial in the inoculum room. In the inoculum room, only the treatment using unsupplemented waste (W10) showed significantly higher results, 5 % higher than the other treatments ($p < 0.05$). The other treatments in the inoculation room show no significant differences in lipidic content ($p \geq 0.05$) (Figure 44).

On the outdoor trial, there were no significant differences in lipid content between the media used by the company (Ino and Org) and the use of supplemented Waste 10 as the culture medium ($p \geq 0.05$) (Figure 44).



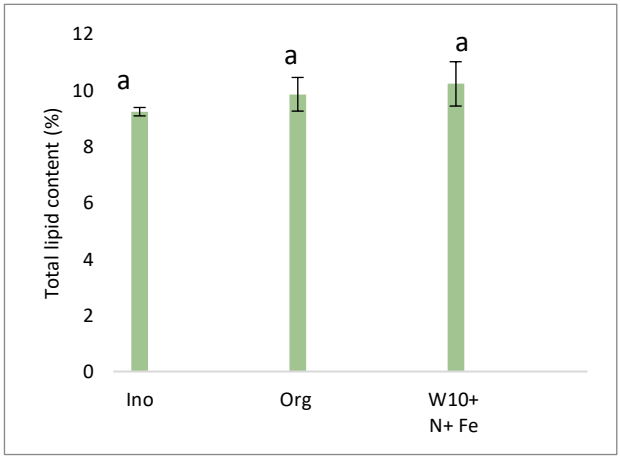


Figure 44: Lipids content (%) of Chlorella biomass produced in inoculum room (3.7) (left) and in outdoors bubble columns (3.8)(right). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and Fe+ M, supplied an iron solution and micronutrients of an inorganic growth solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered ($p < 0.05$).

In the study carried out by Gramegna, the growth of *C. vulgaris* on solid oat and cheese waste was tested. At the end of the study, *C.vulgaris* showed a 17 % lipid content in dry weight. Comparing the values from this study with those obtained herein, the use of **Waste 10** leads to a lower accumulation of lipids. However, in the mentioned study, lipid production was induced through stress during cultivation (Gramegna et al., 2020). In the present study, there was increased lipid production in the non-supplemented W10 condition, which suggests *Chlorella* might have been under stress, most likely nitrogen deprivation.

3.9.3 Ash content

With regard to the organic matter (ash) content, the **Waste 10** condition (non-supplemented), in the inoculum room, had significantly more organic matter than the other treatments ($p < 0.05$). In this treatment it was observed that there was no growth (Figure 36), as it contained more unused inorganic matter (Figure 45).

The results obtained for the ash content in the outdoors bubble columns were similar to those obtained in the inoculum room, taking into account the treatments in common. As a result, there were no significant differences between the treatments using supplemented **Waste 10** (W10+N+ Fe and W10+ N+ Fe+ M) and the company's inorganic culture medium (Ino) ($p \geq 0.05$). On the other hand, the company's organic medium showed significant lower ash content in the inoculum room with the other treatments ($p < 0.05$) and not in the bubble columns ($p \geq 0.05$) (Figure 45).

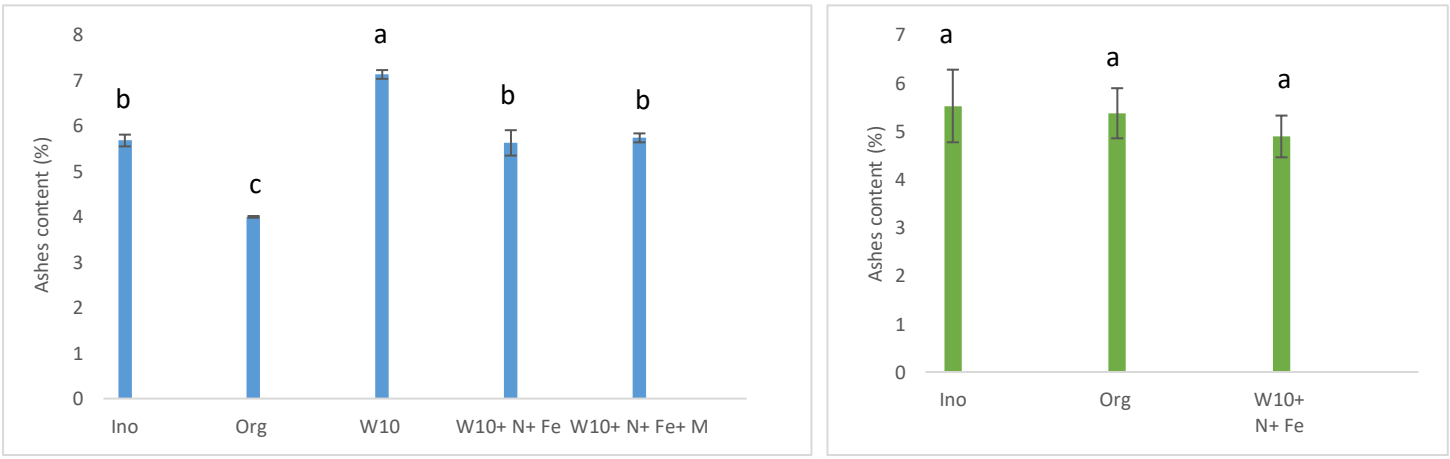


Figure 45: Ashes content (%) of Chlorella biomass produced in inoculum room (3.7) (left) and in outdoors bubble columns (3.8)(right). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and Fe+ M, supplied an iron solution and micronutrients of an inorganic growth solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered ($p < 0.05$).

3.9.4 Carbohydrate content

Looking at the results of the carbohydrate content (Figure 46), different results were attained in the indoor and outdoor trials. In the first test, carried out in the inoculum room, there were significant differences with higher carbohydrates values when using waste as a culture medium than when using the company's media ($p < 0.05$). There were no

differences between the two treatments in which supplemented waste was used ($p \geq 0.05$) (Figure 46).

In the outdoor trial, as was the case indoors, the use of the company organic culture medium showed significantly lower results compared to the carbohydrate content of the other treatments ($p < 0.05$) (Figure 46).

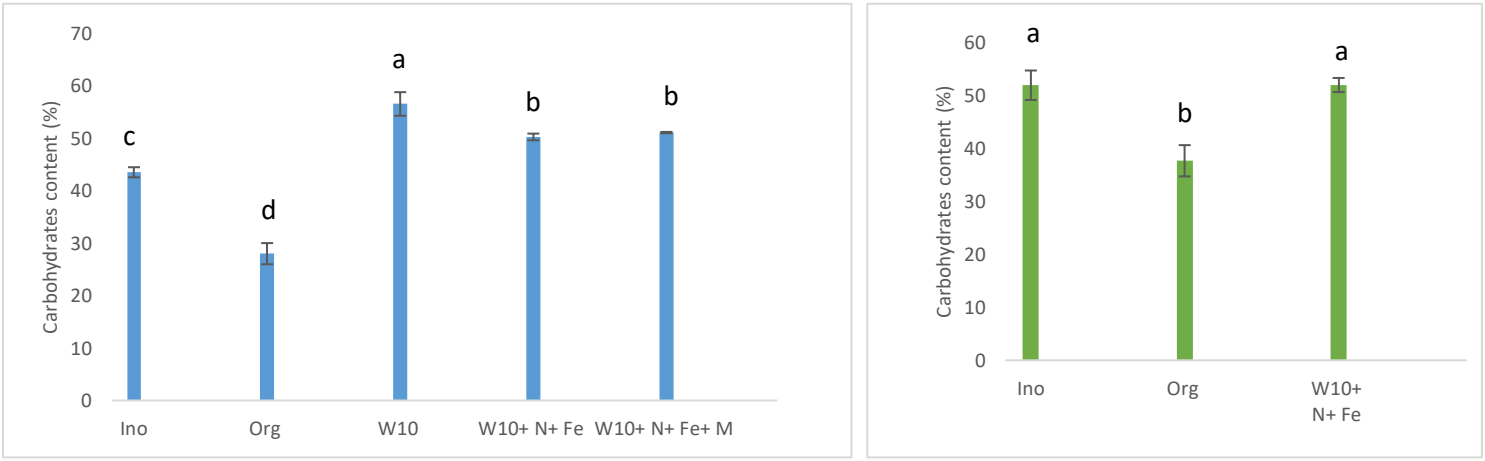


Figure 46: Carbohydrates content (%) of Chlorella biomass produced in inoculum room (3.7) (left) and in outdoors bubble columns (3.8)(right). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and Fe+ M, supplied an iron solution and micronutrients of an inorganic growth solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered ($p < 0.05$).

3.9.5 Pigments

Taking into account the results obtained for chlorophyll (Chl a and b) and total carotenoids in inoculum room (Figure 47), it can be seen that there are no significant differences between the media used by the company and the use of supplemented **Waste 10** ($p \geq 0.05$). **Waste 10** by itself (not supplemented) showed significant differences from the other treatments, with much lower values ($p < 0.05$).

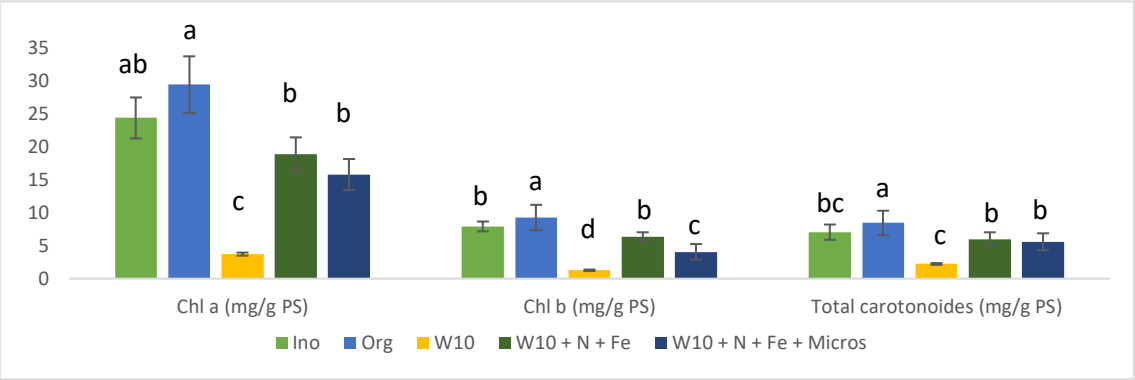


Figure 47: Pigments concentration (mg/ g PS) of Chlorella biomass produced in inoculum room (3.7). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution and Fe+ M, supplied an iron solution and micronutrients of an inorganic growth solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered (p< 0.05).

On outdoors bubble columns (Figure 48), only the treatment in which the company's organic medium was used showed significant lower content of the analysed pigments, comparing with the other treatments (p< 0.05).

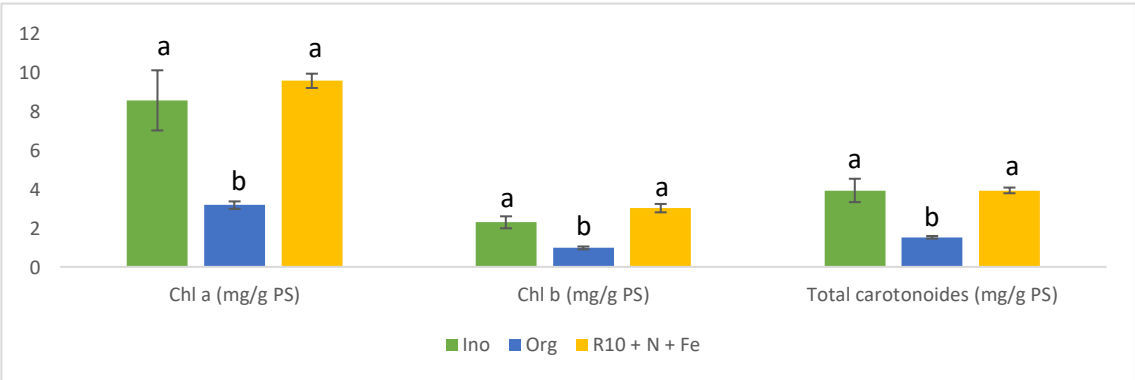


Figure 48: Pigments composition (mg/ g PS) of Chlorella biomass produced in outdoors bubble columns (3.8). Ino and Org represent the industrial medium as a positive control inorganic and organic culture mediums respectively, and the others represent Waste 10 without supplementation (W10) and supplemented with nitrates (N) plus the different micronutrient solutions: Fe, only was supplied iron solution. The results represent the mean of 3 replicates± standard deviation. Letters “a”, “b” and “c” demonstrate significant differences in the different treatments, for each parameter considered (p< 0.05).

This is in agreement with other similar studies found in the literature; for instance, in the study carried out by Vidya, in which the growth of various microalgae was tested in 2 different wastes as a culture media versus a standard medium, the concentration of pigments did not differ significantly in the 3 media analyzed; in this case (as in the present study) the standard medium used (control) is composed of inorganic nutrients (Vidya et al., 2021).

3.10 Spirulina growth test on Waste 10

Finally, a complementary assay was performed, to demonstrate the versatility of the proposed alternative medium solution. In particular, the proposed formulation composed by **Waste 10** supplemented with nitrates was tested as a viable solution for the growth of *Arthrospira platensis*. Besides *Chlorella vulgaris* production, Allmicroalgae is also a large-scale producer of Spirulina and, in line with consumer and market demands, the company is actively looking for organic media solutions for both these species.

Spirulina is a protein-rich species, and it is an extremophile, which renders it competitive advantages, especially relevant for open system cultivation. As previously described, open reactors are generally less sophisticated, and because of its own physiology, spirulina's harvesting and drying can be performed through a more artisanal/manual way, which translates to an overall more economic and environmentally friendly process.

The conventional organic formulation was used as a control, in this case, and **Waste 10** was tested without (W10) or with nitrates supplementation (W10+N), prepared as before (by dissolving 15 g/L of waste in water).

Spirulina's growth curves depicted in Figure 49 suggest lower productivity, compared to *Chlorella*'s (Figure 36). Still, the proposed alternative medium (W10+N) promoted similar productivity than the organic control medium, currently used for industrial production.

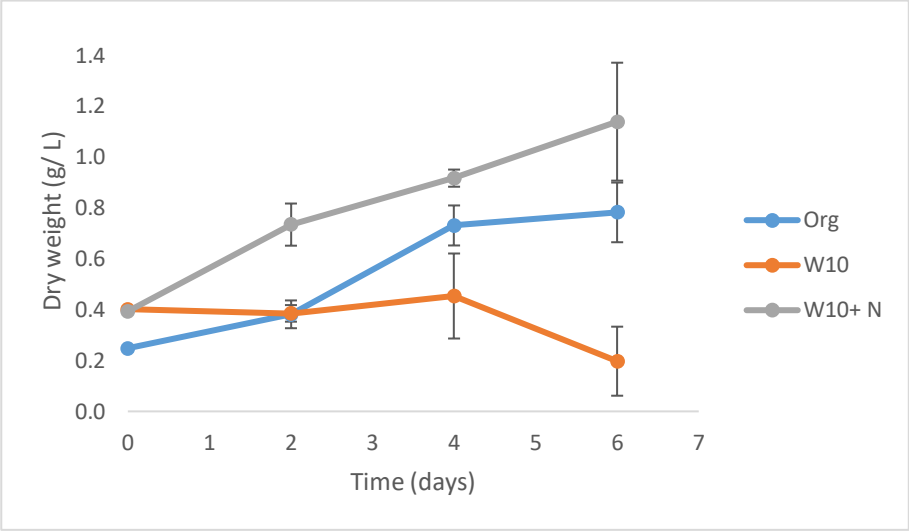


Figure 49: *Arthrospira platensis* growth on Waste 10 at 15 g/ L concentration in the inoculum room. Org represent the industrial medium as a positive control organic culture medium, and the others represents Waste 10 no supplemented (W10) and supplemented with nitrates (W10+ N). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicates± standard deviation.

Overall, W10 by itself did not promote spirulina growth, similarly to previous *Chlorella*'s results, most likely due to nitrogen unavailability. Additionally, there were no significant differences of productivity, comparing the industrially used formulation (Org) to the proposed alternative (W10+N), Figure 50.

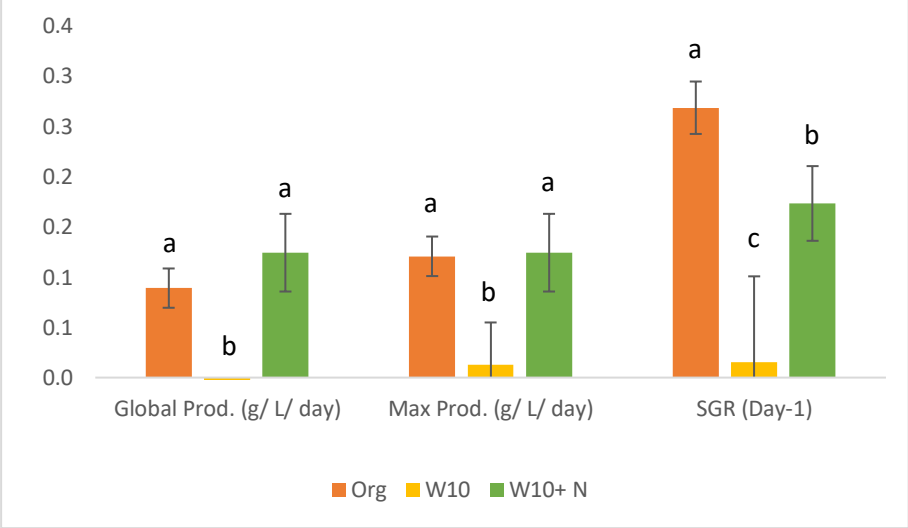


Figure 50: *Arthrospira platensis* growth on Waste 10 at 15 g/ L concentration in the inoculum room. Global Productivity (Global Prod.), Max Productivity (Max Prod.) and Specific growth rate (μ), under the tested conditions. Org represent the industrial medium as a positive control organic culture medium, and the others represents Waste 10 no supplemented (W10) and supplemented with nitrates (W10+ N). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation. Letters "a", "b" and "c" demonstrate significant differences in the different treatments, for each parameter considered ($p< 0.05$).

Still, there was a significant difference of specific growth rate, between the different treatments ($p < 0.05$), with higher values being achieved when the industrial organic medium was used.

Finally, similarly to previous assays with *Chlorella*, nitrogen was consumed as nitrates, but not as urea. Phosphate was consumed throughout Spirulina growth, thus it was only partially consumed in the W10 condition, most likely because the alga did not grow under (presumed) nitrogen deprivation (Figure 51).

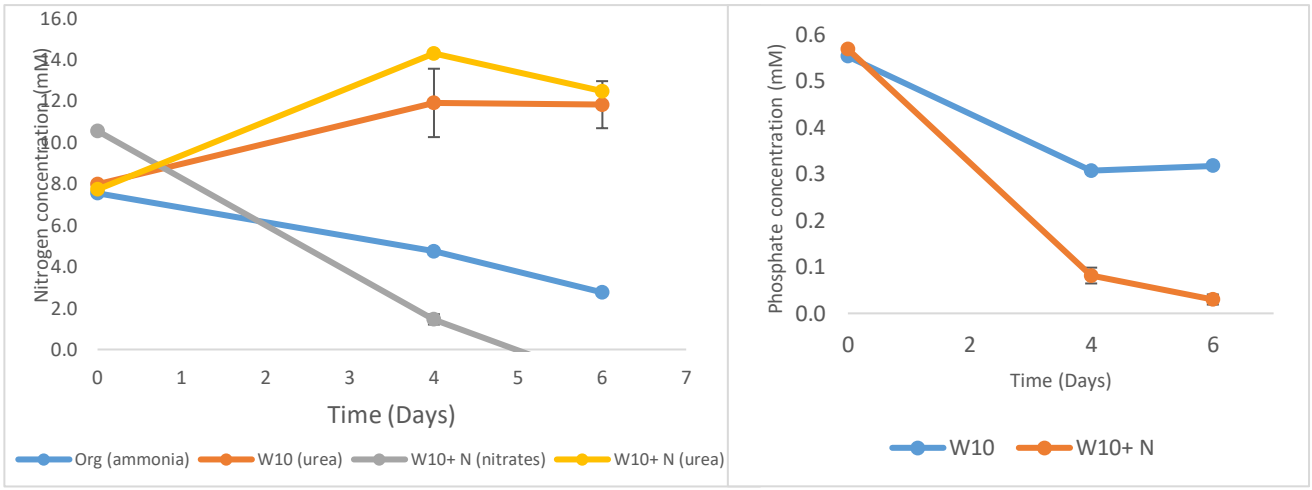


Figure 51: *Arthrospira platensis* consumption of nitrates (left) and urea (right) on the 7 days of trial. Org represents the industrial medium as a positive control organic culture medium, and the others represents Waste 10 no supplemented (W10) and supplemented with nitrates (W10+ N). Culture was grown at an initial dry weight of 0.4 g/L on 1 L reactors at 20º C. The results represent the mean of 3 replicate± standard deviation.

3.11 Tecno-economic analysis

In addition to analyzing growth and biochemistry, it is important to evaluate the costs of using different culture media.

A techno-economic study was therefore carried out, evaluating the costs associated with the latest "Outdoor validation" test, considering water, electricity, CO_2, the cost of the medium itself and supplemented solutions (Table 4). Only the company's culture media (inorganic and organic) acquisition represents a cost for the company, while the acquisition of **Waste 10** would be free of charge. However, the use of **Waste 10** as a culture medium represents an extra cost in its preparation, with energy costs for its homogenization on water.

Considering the cost results obtained, the use of the cultivation method with **Waste 10** showed values close to the use of the inorganic culture medium, so it could easily be replaced, thus reducing food waste. However, the use of the organic medium showed 50% lower costs than the use of the other media, which shows that it is the most economical, although it did not show the best results in terms of growth and pigment content (Table 4).

Table 4: Cost tables for the outdoor test on the bubble columns.

	Water (€)	Electricity (€)	CO_2 (€)	Nitrogen source (€)	Iron (€)	Micronutrients (€)	Total Costs (€)	Total costs / Biomass produced
Inorganic	5	-	7	0.38	0.079	0.031	12.49	6.03
Waste		3.36	3	0.16	0.079	-	11.60	6.39
Organic		-	-	0.29	0.22	0.11	5.62	7.35

When comparing the different mediums, it should also be considered that the company's standard mediums are bought liquid, ready to use, while the use of waste takes into account other costs such as electricity and labour to homogenise it. It is also important, after determining the various results, to find a purpose for the solid waste obtained from filtration and strategies to increase the total bioremediation of waste by microalgae.

The results obtained for the costs per biomass produced (Table 4) show that using the company's organic medium proves to be the most expensive. On the other hand, the company's inorganic medium proves to be the most economical to use to produce *C. vulgaris*. The use of **Waste 10** as a culture medium proved to be slightly more expensive than the company's inorganic medium, but it should be noted that it could still be optimized and is very promising as a replacement for the company's inorganic medium.

According to the company's *C. vulgaris* production method, the inorganic medium is used in the initial microalgae scale-up phase and, when the culture is transferred to photobioreactors (with large production volumes), organic medium is used. In this way, the use of **Waste 10** could be a promising substitute for the inorganic medium, with no implications for production and a reduction in food waste.

Conclusion

Reducing food waste is a pressing issue demanding swift action and innovative solutions. One promising approach to promote a circular economy, generate employment opportunities, produce valuable chemicals, and reduce the environmental impact of waste involves harnessing microalgae to recycle and enhance the value of food remnants. The capacity of microalgae to remediate food waste presents a unique opportunity to advance sustainability and food security, benefiting both current and future generations.

However, to guarantee an equitable shift towards sustainable food systems that benefit the entire community, it is essential to institute policies and practices that are inclusive and fair. Governments have a crucial role to play in crafting legal structures that support endeavours aimed at averting and reducing food waste. This requires cooperative initiatives spanning multiple policy domains, as well as the implementation of financial incentives to stimulate the adoption of waste reduction strategies.

With this work, it was possible to demonstrate a method of cultivating microalgae with a culture medium based on food waste (**Waste 10**). The data obtained shows that *C. vulgaris* can be grown outdoors with the use of waste 10 supplemented with nitrated and iron as culture medium, while maintaining on the same productivity and biochemical composition, of the culture grown with inorganic culture medium used by the company. However, further research into this method is crucial to minimize costs and make better use of the nutrients present in the waste, especially the nitrogen source.

References

Abreu AP, Fernandes B, Vicente AA, Teixeira J, Dragone G (2012) Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. Bioresource technology **118:** 61-66

Ahmad MT, Shariff M, Md. Yusoff F, Goh YM, Banerjee S (2020) Applications of microalga Chlorella vulgaris in aquaculture. Reviews in Aquaculture **12:** 328-346

Andersen RA (2005) Algal culturing techniques. Elsevier

Armstrong F (1963) Determination of Nitrate in Water Ultraviolet Spectrophotometry. Analytical Chemistry **35:** 1292-1294

Aschemann-Witzel J, Giménez A, Ares G (2018) Consumer in-store choice of suboptimal food to avoid food waste: The role of food category, communication and perception of quality dimensions. Food Quality and Preference **68:** 29-39

Barrera EL, Hertel T (2021) Global food waste across the income spectrum: Implications for food prices, production and resource use. Food Policy **98:** 101874

Barros A, Pereira H, Campos J, Marques A, Varela J, Silva J (2019) Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large scale production of microalgae. Scientific Reports **9:** 13935

Bhatia L, Jha H, Sarkar T, Sarangi PK (2023) Food waste utilization for reducing carbon footprints towards sustainable and cleaner environment: a review. International Journal of Environmental Research and Public Health **20:** 2318

Blas-Valdivia V, Ortiz-Butrón R, Pineda-Reynoso M, Hernández-Garcia A, Cano-Europa E (2011) Chlorella vulgaris administration prevents HgCl 2-caused oxidative stress and cellular damage in the kidney. Journal of Applied Phycology **23:** 53-58

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology **37:** 911-917

Borowitzka MA (1992) Algal biotechnology products and processes—matching science and economics. Journal of applied phycology **4:** 267-279

Brancoli P, Rousta K, Bolton K (2017) Life cycle assessment of supermarket food waste. Resources, Conservation and Recycling **118:** 39-46

Bux F (2013) Biotechnological applications of microalgae: biodiesel and value-added products. CRC Press

Cheirsilp B, Suwannarat W, Niyomdecha R (2011) Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock. New Biotechnology **28:** 362-368

Chen H, Zheng Y, Zhan J, He C, Wang Q (2017) Comparative metabolic profiling of the lipid-producing green microalga Chlorella reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism. Biotechnology for biofuels **10:** 1-20

Chia MA, Lombardi AT, Maria da Graça GM, Parrish CC (2013) Lipid composition of Chlorella vulgaris (Trebouxiophyceae) as a function of different cadmium and phosphate concentrations. Aquatic toxicology **128:** 171-182

Chia MA, Lombardi AT, Melão MDGG, Parrish CC (2013) Effects of cadmium and nitrogen on lipid composition of Chlorella vulgaris (Trebouxiophyceae, Chlorophyta). European Journal of Phycology **48:** 1-11

Chinnasamy S, Bhatnagar A, Claxton R, Das K (2010) Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresource technology **101:** 6751-6760

Chisti Y (2018) Society and microalgae: Understanding the past and present. *In* Microalgae in health and disease prevention. Elsevier, pp 11-21

de Carvalho JC, Sydney EB, Tessari LFA, Soccol CR (2019) Culture media for mass production of microalgae. *In* Biofuels from algae. Elsevier, pp 33-50

de Medeiros VPB, Pimentel TC, Varandas RCR, Dos Santos SA, de Souza Pedrosa GT, da Costa Sassi CF, da Conceição MM, Magnani M (2020) Exploiting the use of agro-industrial residues from fruit and vegetables as alternative microalgae culture medium. Food Research International **137:** 109722

Delilah D, Mathew NM, Joseph A, Jose S, Kumar S (2022) Plant Micronutrient Nanoparticles from Microalgae-Biosynthesis and their Applications–A Review. Int. J. Bot. Stud **7:** 135-139

El-Fadel M, Findikakis AN, Leckie JO (1997) Environmental impacts of solid waste landfilling. Journal of environmental management **50:** 1-25

Felix C, Ubando A, Madrazo C, Sutanto S, Tran-Nguyen PL, Go AW, Ju Y-H, Culaba A, Chang J-S, Chen W-H (2019) Investigation of direct biodiesel production from wet microalgae using definitive screening design. Energy Procedia **158:** 1149-1154

Feng Y, Li C, Zhang D (2011) Lipid production of Chlorella vulgaris cultured in artificial wastewater medium. Bioresource technology **102:** 101-105

Flynn KJ (1990) The determination of nitrogen status in microalgae. Marine ecology progress series. Oldendorf **61:** 297-307

Gramegna G, Scortica A, Scafati V, Ferella F, Gurrieri L, Giovannoni M, Bassi R, Sparla F, Mattei B, Benedetti M (2020) Exploring the potential of microalgae in the recycling of dairy wastes. Bioresource Technology Reports **12:** 100604

Grobbelaar JU (2004) Algal nutrition: mineral nutrition. Handbook of microalgal culture: biotechnology and applied phycology**:** 97-115

Jin H, Zhang H, Zhou Z, Li K, Hou G, Xu Q, Chuai W, Zhang C, Han D, Hu Q (2020) Ultrahigh-cell-density heterotrophic cultivation of the unicellular green microalga Scenedesmus acuminatus and application of the cells to photoautotrophic culture enhance biomass and lipid production. Biotechnology and Bioengineering **117:** 96-108

Khan S, Khan M, Hanjra MA, Mu J (2009) Pathways to reduce the environmental footprints of water and energy inputs in food production. Food policy **34:** 141-149

Kolev Slavov A (2017) Pregled općih svojstava i mogućih postupaka obrade otpadnih voda mljekarske industrije. Food Technology and Biotechnology **55:** 14-28

Kong W, Song H, Cao Y, Yang H, Hua S, Xia C (2011) The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of Chlorella vulgaris under mixotrophic cultivation. African Journal of Biotechnology **10:** 11620-11630

Kothari R, Pathak VV, Kumar V, Singh D (2012) Experimental study for growth potential of unicellular alga Chlorella pyrenoidosa on dairy waste water: an integrated approach for treatment and biofuel production. Bioresource technology **116:** 466-470

Leung CCJ, Cheung ASY, Zhang AY-Z, Lam KF, Lin CSK (2012) Utilisation of waste bread for fermentative succinic acid production. Biochemical engineering journal **65:** 10-15

Li K, Liu Q, Fang F, Luo R, Lu Q, Zhou W, Huo S, Cheng P, Liu J, Addy M (2019) Microalgae-based wastewater treatment for nutrients recovery: A review. Bioresource technology **291:** 121934

Li T, Zheng Y, Yu L, Chen S (2013) High productivity cultivation of a heat-resistant microalga Chlorella sorokiniana for biofuel production. Bioresource technology **131:** 60-67

Mao X, Zhou X, Fan X, Jin W, Xi J, Tu R, Naushad M, Li X, Liu H, Wang Q (2023) Proteomic analysis reveals mechanisms of mixed wastewater with different N/P ratios affecting the growth and biochemical characteristics of Chlorella pyrenoidosa. Bioresource Technology **381:** 129141

Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews **14:** 217-232

Melikoglu M, Lin CSK, Webb C (2013) Analysing global food waste problem: pinpointing the facts and estimating the energy content. Central European Journal of Engineering **3:** 157-164

Molazadeh M, Danesh S, Ahmadzadeh H, Pourianfar HR (2019) Influence of CO2 concentration and N: P ratio on Chlorella vulgaris-assisted nutrient bioremediation, CO2 biofixation and biomass production in a lagoon treatment plant. Journal of the Taiwan Institute of Chemical Engineers **96:** 114-120

Moseley J, Grossman AR (2009) Phosphate metabolism and responses to phosphorus deficiency. *In* The Chlamydomonas Sourcebook. Elsevier, pp 189-215

Murphy CF, Allen DT (2011) Energy-water nexus for mass cultivation of algae. Environmental science & technology **45:** 5861-5868

Narala RR, Garg S, Sharma KK, Thomas-Hall SR, Deme M, Li Y, Schenk PM (2016) Comparison of microalgae cultivation in photobioreactor, open raceway pond, and a two-stage hybrid system. Frontiers in Energy Research **4:** 29

Ng DH, Ng YK, Shen H, Lee YK (2015) Microalgal biotechnology: The way forward. *In* Handbook of marine microalgae. Elsevier, pp 69-80

Nunez M, Quigg A (2016) Changes in growth and composition of the marine microalgae Phaeodactylum tricornutum and Nannochloropsis salina in response to changing sodium bicarbonate concentrations. Journal of Applied Phycology **28:** 2123-2138

Pozzobon V, Cui N, Moreaud A, Michiels E, Levasseur W (2021) Nitrate and nitrite as mixed source of nitrogen for Chlorella vulgaris: Growth, nitrogen uptake and pigment contents. Bioresource Technology **330:** 124995

Procházková G, Brányiková I, Zachleder V, Brányik T (2014) Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. Journal of applied phycology **26:** 1359-1377

Pulz O, Scheibenbogen K, Groß W (2001) Biotechnology with cyanobacteria and microalgae. Biotechnology Set**:** 105-136

Quigg A (2016) Micronutrients. The physiology of microalgae**:** 211-231

Rai MP, Gautom T, Sharma N (2015) Effect of salinity, pH, light intensity on growth and lipid production of microalgae for bioenergy application. OnLine Journal of Biological Sciences **15:** 260

Rajanren JR, Ismail HM (2017) Investigation of Chlorella vulgaris microalgae as a source for renewable fuel. Biofuels **8:** 37-47

Richmond A (2004) Handbook of microalgal culture: biotechnology and applied phycology, Vol 577. Wiley Online Library

Richmond A, Hu Q (2013) Handbook of microalgal culture. Wiley Online Library

Rizwan M, Mujtaba G, Memon SA, Lee K, Rashid N (2018) Exploring the potential of microalgae for new biotechnology applications and beyond: A review. Renewable and Sustainable Energy Reviews **92:** 394-404

Rodriguez-Garcia I, Guil-Guerrero JL (2008) Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food chemistry **108:** 1023-1026

Roijers A, Tas M (1964) The determination of urea with p-dimethylaminobenzaldehyde. Clinica Chimica Acta **9:** 197-202

Safi C, Zebib B, Merah O, Pontalier P-Y, Vaca-Garcia C (2014) Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. Renewable and Sustainable Energy Reviews **35:** 265-278

Sayeki M, Kitagawa T, Matsumoto M, Nishiyama A, Miyoshi K, Mochizuki M, Takasu A, Abe A (2001) Chemical composition and energy value of dried meal from food waste as feedstuff in swine and cattle. Animal Science Journal (Japan)

Schmidt K, Matthies E (2018) Where to start fighting the food waste problem? Identifying most promising entry points for intervention programs to reduce household food waste and overconsumption of food. Resources, Conservation and Recycling **139:** 1-14

Schmitz R, Magro CD, Colla LM (2012) Aplicações ambientais de microalgas. Revista CIATEC-UPF **4:** 48-60

Serra-Maia R, Bernard O, Gonçalves A, Bensalem S, Lopes F (2016) Influence of temperature on Chlorella vulgaris growth and mortality rates in a photobioreactor. Algal research **18:** 352-359

Shibata S, Sansawa H (2006) Preventive effects of heterotrophically cultured Chlorella regularis on lifestyle-associated diseases. Annu. Rep. Yakult Central Inst. Microbiol. Res **26:** 63-72

Siddiki SYA, Mofijur M, Kumar PS, Ahmed SF, Inayat A, Kusumo F, Badruddin IA, Khan TY, Nghiem L, Ong HC (2022) Microalgae biomass as a sustainable source for biofuel, biochemical and biobased value-added products: An integrated biorefinery concept. Fuel **307:** 121782

Subhash GV, Rajvanshi M, Kumar BN, Govindachary S, Prasad V, Dasgupta S (2017) Carbon streaming in microalgae: extraction and analysis methods for high value compounds. Bioresource technology **244:** 1304-1316

Suganya T, Varman M, Masjuki H, Renganathan S (2016) Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. Renewable and Sustainable Energy Reviews **55:** 909-941

Udayan A, Arumugam M, Pandey A (2017) Nutraceuticals from algae and cyanobacteria. *In* Algal green chemistry. Elsevier, pp 65-89

Vidya D, Nayana K, Sreelakshmi M, Keerthi K, Mohan KS, Sudhakar M, Arunkumar K (2021) A sustainable cultivation of microalgae using dairy and fish wastes for enhanced biomass and bio-product production. Biomass Conversion and Biorefinery**:** 1-15

Vuppaladadiyam AK, Prinsen P, Raheem A, Luque R, Zhao M (2018) Microalgae cultivation and metabolites production: a comprehensive review. Biofuels, Bioproducts and Biorefining **12:** 304-324

Wu LF, Chen PC, Huang AP, Lee CM (2012) The feasibility of biodiesel production by microalgae using industrial wastewater. Bioresource Technology **113:** 14-18

Yamamoto M, Fujishita M, Hirata A, Kawano S (2004) Regeneration and maturation of daughter cell walls in the autospore-forming green alga Chlorella vulgaris (Chlorophyta, Trebouxiophyceae). Journal of plant research **117:** 257-264

Zhang AY-z, Sun Z, Leung CCJ, Han W, Lau KY, Li M, Lin CSK (2013) Valorisation of bakery waste for succinic acid production. Green chemistry **15:** 690-695

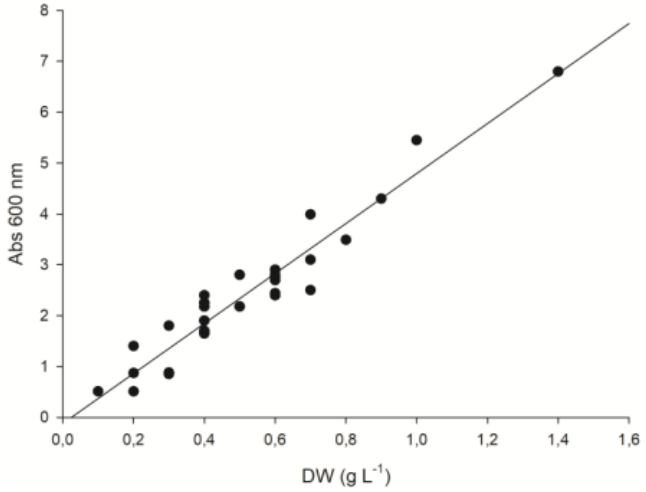
Appendix

A. Composition of the different wastes tested

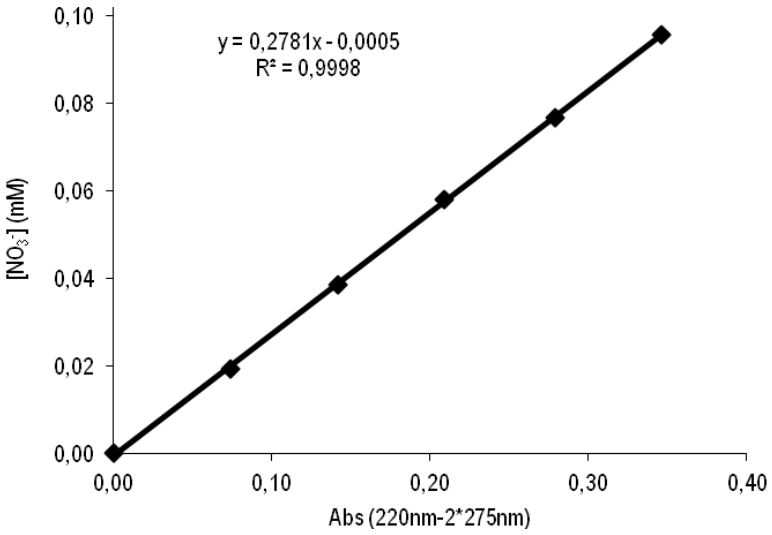
Waste	Fruits	Vegetables	Dairy products	Bakeries	Meat	Fish	Sawdust	Final appearance
7	(10 Kg) Bananas+ Apple+ Oranges	(10 Kg) Carrot+ Pumpkin+ Stalk	(1kg) Solid yoghurt	(12kg) Traditional bread	-	(7kg) Frozen fish	-	Dry powder
10	(10 Kg) Banana+ Strawberry+ Grapefruit	(10 Kg) Pumpkin+ Lettuce+ Kale+ Cucumber	(6kg) Liquid + solid yoghurts	(12kg) Traditional bread	-	(7kg) Frozen fish	-	Dry powder
12	(10 Kg) Banana+ Mango+ Orange+ Strawberry	(10 Kg) Pumpkin+ Garlic+ Mushrooms	(6kg) Milk + solid yoghurt	(12kg) Traditional bread	(7kg) Non-frozen meat	-	-	Dry powder
13	(30 Kg) Banana+ Mango+ Orange	(30 Kg) Onion+ Potato+ various vegetables	(18kg) Liquid + solid yoghurts	(12kg) Traditional bread	-	-	-	Powder
15	(10 kg) Orange+ Pear+ Lemon+ Meloa	(10 Kg) Potato+ Cabbage+ Lettuce+ Peppers	(6 Kg) Solid yoghurt	-	-	(7kg) Frozen fish	(12Kg) Sawdust	Powder
16	(30 Kg) Banana+ Mango+ Orange+ Strawberry	(30Kg) Tomate+ Alface+ Courgette+ Repolho	(18 Kg) Solid+ liquid yoghurts	-	-	-	(12Kg) Sawdust	Half-moist powder
17	(10 Kg) Banana+ Pear+ Apple+ Orange	(10 Kg) Potato+ Tomato+ Lettuce	(6 Kg) Liquid yoghurts+ Milk	-	(7kg) Non-frozen meat	-	(12Kg) Sawdust	Dry powder

B. Calibration curves

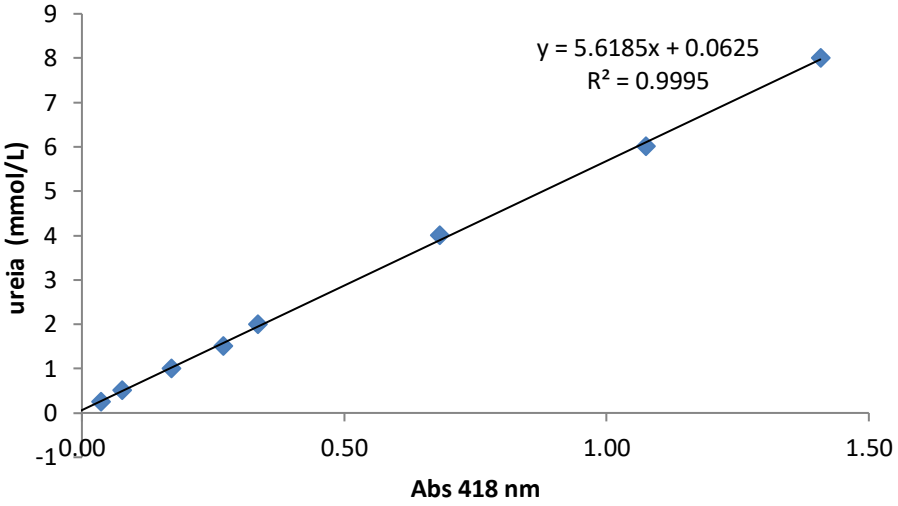
1. Absorbance of Chlorella sp. measured at λ = 600 nm vs dry biomass concentration for autotrophic growth



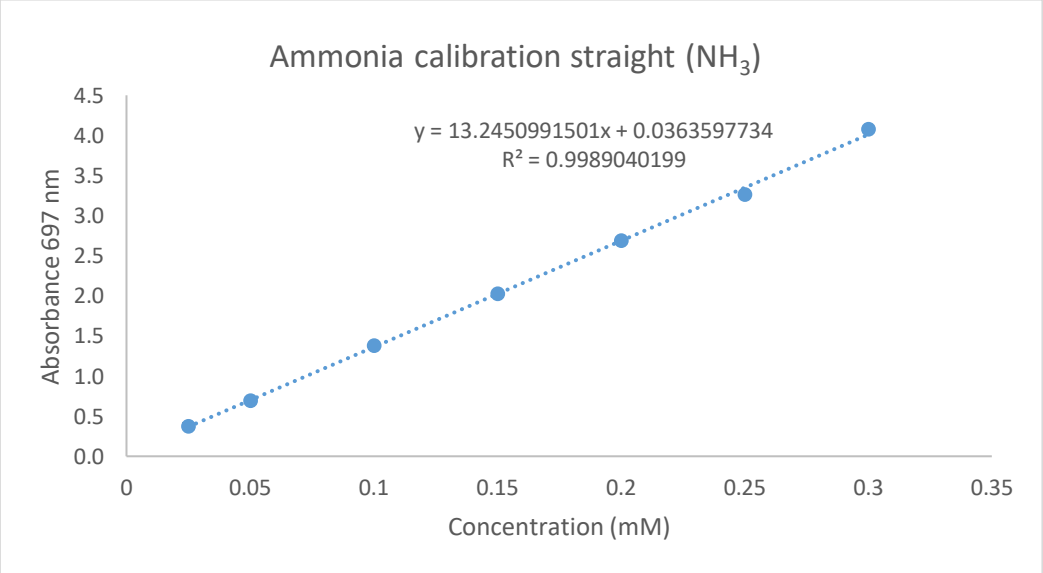
2. Concentration of nitrates



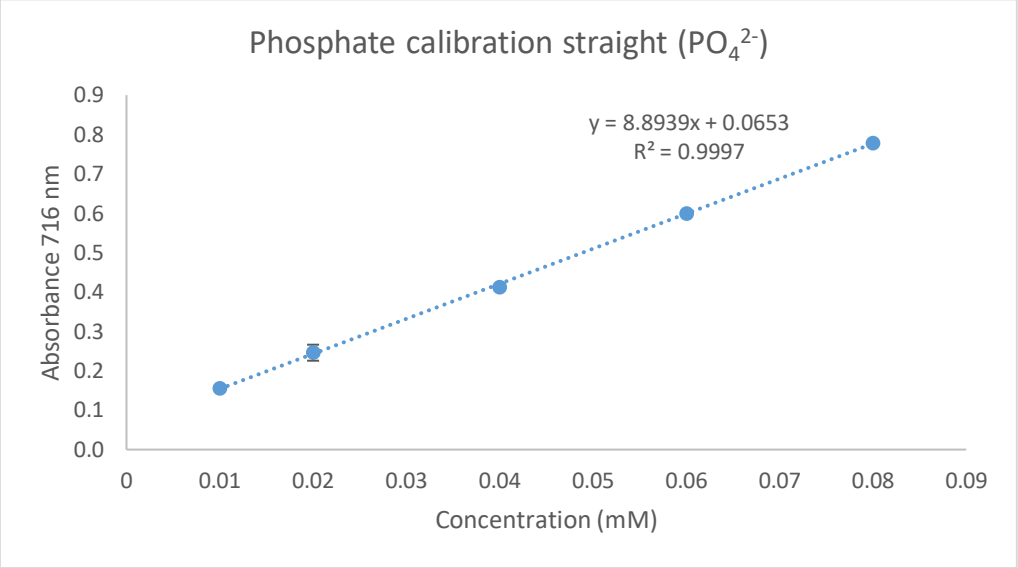
3.Concentration of urea



4. Concentration of ammonium



5. Concentration of phosphate



6. Concentration of iron