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Oxyfluorfen: a novel metabolic inhibitor to select microalgal chlorophyll-deficient mutant strains for nutritional applications

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A R T I C L E I N F O

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A B S T R A C T

Nowadays, there is an increasing demand for novel feedstocks and alternative protein sources to meet global needs. Because of their rich nutritional profiles and high protein contents, microalgae-based food products and supplements are being developed. Nonetheless, these products present organoleptic characteristics such as taste, smell and colour that are often considered unpleasant by human and animal consumers. To address this constraint, strain improvement approaches such as random mutagenesis have been used, which combined with the right selection strategy, lead to more appealing microalgal biomass. In this work, a novel selection strategy using oxyfluorfen, an inhibitor of the chlorophyll synthesis pathway, was applied for the first time to isolate chlorophyll-deficient strains of *Scenedesmus rubescens* and *Chlorella vulgaris* upon treatment with ethyl methanesulfonate (EMS). With this approach, one *S. rubescens* brownish (37Y01) mutant strain, as well as two *C. vulgaris* mutant strains, one yellow (31Y15) and one white (31W62), were obtained. *S. rubescens* 37Y01 displayed a reduced protein content of 19.1% dry weight (DW) compared to that of the wildtype, which presented a protein content of 25.0% DW. *C. vulgaris* wildtype and mutants exhibited higher protein contents, in the 42.8-44.3% DW range, compared to *Scenedesmus rubescens* ($p < 0.05$). The selective pressure of this inhibitor allowed the selection of *S. rubescens* and *C. vulgaris* mutants displaying 55% and 95% decrease in chlorophyll content, respectively. The reduced chlorophyll content greatly improves the sensory properties and consumer acceptance of established mutants, increasing the potential of both strains as feedstocks to develop novel food products.

1. Introduction

Global demand for food and feed has been increasing steadily, along with the world population (www.un.org/en/global-issues/population), resulting in a progressively compromised food security worldwide, particularly regarding protein supply [1,2]. Thus, it is urgent to explore

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alternatives to conventional feedstocks and adapt the food system towards more sustainable, healthy and affordable diets [3]. In this context, microalgae have been a subject of greater interest as one of the promising alternatives for food and feed and other biotechnological applications [4].

Microalgae are natural sources of proteins, lipids and carbohydrates, with diverse and complete nutritional profiles [4–6]. They are also a source of vitamins, pigments, bioactive compounds and high-quality vegan protein with all the essential amino acids [7].

However, microalgae-based industries have been facing multiple challenges. Despite all the investment in R&D, microalgal products struggle to reach competitive market prices, mainly due to low biomass and target compounds productivities as well as costly inputs [8–10]. The organoleptic characteristics of the biomass, namely the green colour and grassy taste originated by chlorophyll, also hinder consumers' and even animals' acceptance [11–15]. Finally, only a limited number of microalgal strains, such as *Chlorella* spp., are currently approved as novel foods in Europe (www.algae-novel-food.com/output/algae-novel-food/download.pdf) (EU, 2017/2470; EU, 2015/2283).

Overcoming the current bottlenecks of microalgae production requires a multistage optimization approach in the whole cultivation and processing pipeline, starting with strain selection and improvement [16–19]. Several strategies have been applied to generate and select mutants suitable for large-scale cultivation, such as adaptive laboratory evolution, random mutagenesis and gene editing tools [19–21]. A more detailed discussion of these methodologies is available in a recent review by Trovão et al. [21].

Random mutagenesis is a well-established and cost-effective technology that accelerates the natural occurrence of spontaneous mutations [13,17,21,22]. Unlike insertional mutagenesis, random mutagenesis does not introduce foreign genetic material into the cells, allowing to readily generate a wide variety of mutant phenotypes without the restrictions imposed on genetically modified organisms (GMOs) [21–24]. This technology enables the isolation of more productive and tolerant microalgal strains for different biotechnological applications [21,25]. However, there are two limiting steps concerning random mutagenesis: lack of effective selection methods for different characteristics and phenotype instability [21,25,26].

Several selection strategies have been developed to select a target phenotype out of numerous mutant colonies. Methods based on the characterization of each colony, such as visual appearance and autofluorescence, are often used to select fast-growing and differently coloured mutants. Nevertheless, these techniques are heavily time-consuming and hamper the reduction of the number of colonies to test. High-throughput technologies, such as fluorescence-activated cell sorting (FACS), are promising tools to address this lack of efficiency but still require further innovation and study to take full advantage of it [17,22,25,27]. Exposure to stressful conditions is also a commonly used approach to select mutants with tolerant phenotypes [21]. Finally, a more direct approach might be applied to select mutants producers of specific biocompounds by resorting to pathway inhibitors that target enzymes or regulatory factors involved in the biosynthetic pathway of the compounds of interest, such as carotenoids [21,28,29]. Although the latter enables the isolation of mutants with improved biochemical profiles, these mutations often hamper or are detrimental to growth due to a loss of function. In addition, mutants might revert their phenotype, either through DNA damage repair mechanisms or secondary mutations [30,31]. Thus, new selection strategies, namely using new inhibitors that directly target specific pathways, should be studied to select improved phenotypes and prevent their reversion.

In this context, oxyfluorfen, an inhibitor that specifically targets the chlorophyll biosynthetic pathway [32,33], holds high potential to isolate stable chlorophyll-deficient mutants with different pigmentation profiles. Oxyfluorfen is a diphenyl ether that has been used as an herbicide in agriculture since 1976 [34,35]. This fluorinated chemical was reported to inhibit protoporphyrinogen oxidase (PPO) by inducing the formation of reactive oxygen species (ROS) through the interaction of oxygen with protoporphyrin IX, which leads to the inhibition of the synthesis of photosynthetic pigments as chlorophyll (Fig. 1) [32–36].

Therefore, this work focused on the optimization of a novel strain improvement pipeline using oxyfluorfen to establish chlorophyll-deficient mutants from *Chlorella vulgaris* and *Scenedesmus rubescens* with protein contents similar to the wildtype strains but with improved organoleptic characteristics. To the best of our knowledge, this is the first report of using oxyfluorfen as a selective inhibitor upon random mutagenesis with ethyl methanesulfonate (EMS) to isolate improved mutants of *C. vulgaris* and *S. rubescens*. Moreover, the mutagenesis for altered pigment profiles with EMS is here described for the first time for the genus *Scenedesmus*.

2. Materials and methods

2.1. Wildtype inocula and culture media

Scenedesmus rubescens and *Chlorella vulgaris* axenic cultures were obtained from Allmicroalgae Natural Products S.A. culture collection. The wildtype seeds were obtained from cryopreserved aliquots stored in liquid nitrogen (−196 °C).

The optimized medium reported by Espírito Santo et al. [39] was used to cultivate *S. rubescens* with a modification of the nitrogen source (Sr-HM): sodium nitrate (10 mM) and urea (30 mM) were used instead of ammonia. The heterotrophic medium (HM) described by Barros et al. [40] was used to cultivate *C. vulgaris* (Cv-HM), with 20 mM of ammonium sulphate. For both species, glucose at 20 g L^{-1} was used as the carbon source.

2.2. EMS dose-response experiments

According to the protocol described by Schüler et al. [13], WT inocula in the early exponential phase were concentrated 10-fold in the respective Sr-HM or Cv-HM medium by centrifugation. Concentrated *S. rubescens* culture (1.54×10^6 cells mL^{-1}) was treated with 0–600 mM of EMS (Sigma-Aldrich, St. Louis, USA) and incubated under constant agitation (100 rpm) at 28 °C for 1 h in the dark. Similarly, *C. vulgaris* culture (2.16×10^5 cells mL^{-1}) was exposed to 0–300 mM of EMS. The cells were incubated at 30 °C and 100 rpm for 1 h in the dark. The EMS reaction was stopped by adding sodium thiosulfate (5%, w/w) and cells were resuspended in the respective diluted HM medium (1:2) after washing thrice. The mutagenized cells were incubated for 24 h in the dark at 28 °C and 30 °C, respectively, for *S. rubescens* and *C. vulgaris*. In order to determine the survival rate, cultures were plated in triplicates onto Plate Count Agar (PCA; HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated in the dark at the corresponding growth temperatures for 15–21 days.

2.3. Oxyfluorfen toxicity

Scenedesmus rubescens (1.01×10^6 cells mL^{-1}) and *Chlorella vulgaris* (2.16×10^5 cells mL^{-1}) in early exponential phase were concentrated 10-fold in the respective diluted HM medium (1:2) by centrifugation. Then, 0.1 mL of concentrated culture was spread onto 6-well plates containing PCA supplemented with oxyfluorfen. All conditions were tested in triplicates. Oxyfluorfen solution in methanol was prepared at 500 mg L^{-1}, which was filter-sterilized with 0.22 μm PTFE filters (Labbox Labware, SL, Barcelona, Spain). Oxyfluorfen was tested at 0–700 μg L^{-1} for *S. rubescens* and at 0–5000 μg L^{-1} for *C. vulgaris*.

Plates were incubated in the dark at the respective growth temperatures for 14 days, after which the growth in each well was observed and compared to the control. In addition, a sample from each condition was replated onto PCA without the inhibitor. After 3–5 days, the growth was visually analysed to determine the optimal concentration range of oxyfluorfen to be used in mutant selection.

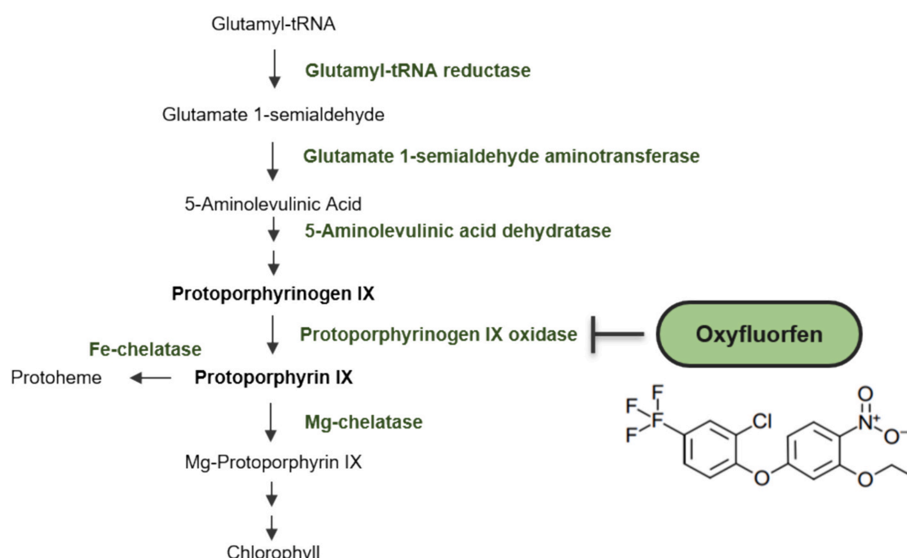

Fig. 1. Simplified chlorophyll synthesis pathway and inhibition target of oxyfluorfen. Adapted from [33,37,38].

2.4. Random mutagenesis and mutants' selection

According to the dose-response curves previously established (Section 2.2), random mutagenesis was performed on *S. rubescens* (1.54×10^6 cells mL^{-1}) with 150, 200 and 250 mM of EMS, while for *C. vulgaris* (2.16×10^5 cells mL^{-1}) the EMS concentrations used were 100, 125 and 150 mM. The mutagenesis protocol was as described above (Section 2.2).

Upon mutagenesis, a selection step was applied by resorting to oxyfluorfen. *S. rubescens* mutagenized cells were plated onto PCA with 100, 200 or 300 μg L^{-1} of oxyfluorfen and incubated in the dark at 28 °C for 28 days. For selecting *C. vulgaris* mutants, the cultures were plated onto PCA with 300, 400 and 500 μg L^{-1} of oxyfluorfen and incubated in the dark at 30 °C for 28 days. For each plate, 0.1 mL of concentrated culture was spread. Differently coloured colonies were picked and streaked onto PCA with the same concentration of oxyfluorfen and re-streaked for 10 generations to ensure phenotype stability. A pre-selection step was carried out, in which 9 yellow and 11 white mutants of *C. vulgaris* and 4 brownish mutants of *S. rubescens* were isolated. These mutants were streaked several times in plate, as explained, and only one of each species was selected, with stable phenotype and the most appealing colour.

2.5. Growth performance of wildtype vs mutants

Mutants with a stable phenotype on solid medium were transferred to liquid medium (without inhibitor) to compare their growth performance with the respective wildtype strains. Growth assays were conducted in 250-mL Erlenmeyer flasks in triplicate with a final working volume of 50 mL.

Scenedesmus rubescens WT (37WT) and its mutants were grown at 28 °C, in Sr-HM medium, at pH 6.5, with PIPES buffer at 60 mM, in an orbital incubator set at 200 rpm (ArgoLab® shaker SKI 4, Carpi, Italy). Likewise, *C. vulgaris* WT (31WT) and its mutants were cultivated at 30 °C, 200 rpm, in Cv-HM medium, at pH 6.5, with PIPES buffer at 50 mM.

Cultures were sampled and analysed daily by measuring the optical density at 600 nm (OD_{600}) (Genesys 10S UV–Vis®; Thermo Fisher Scientific, Massachusetts, EUA) and medium pH (Metria universal pH test paper strips; Labbox Labware, SL, Barcelona, Spain), as well as through optical microscopy (Axio Scope A1®, Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Dry weight (DW) was determined by filtering microalgal suspensions using pre-weighed 0.7 μm glass microfibre filters (VWR International, Pennsylvania, USA) and washed with demineralized water. Finally, the samples were dried at 120 °C and weighed using a moisture analyzer (MA 50.R Moisture Analyzer, Radwag®, Radom, Poland). The DW was calculated as follows:

$$DW\ (g\ L^{-1}) = \frac{(m_f - m_i)}{V} \quad (1)$$

where m_f - m_i represent the algal mass collected by filtration and V corresponds to the volume of filtrated cell suspension.

A DW vs. OD correlation was established for *S. rubescens* (Eq. (2); $R^2 = 0.8478$) and *C. vulgaris* (Eq. (3); $R^2 = 0.9136$):

$$OD\ (600\ nm) = (0.5566 \times DW) + 1.416 \quad (2)$$

$$OD\ (600\ nm) = (0.5934 \times DW) \quad (3)$$

The growth rate was obtained by Eq. (4) and biomass productivity by Eq. (5), where DW_f and DW_i correspond to the final and initial dry weights measured at the time points t_f and t_i, respectively.

$$\mu\ (day^{-1}) = \frac{ln(DW_f/DW_i)}{(t_f - t_i)} \quad (4)$$

$$P(g\ L^{-1} day^{-1}) = \frac{(DW_f - DW_i)}{(t_f - t_i)} \quad (5)$$

At the end of each assay, samples were centrifuged at 4500 *g* for 15 min (Hermle® Z300 centrifuge, Gosheim, Germany), and pellets were frozen at −20 °C, freeze-dried in a Coolvacuum, Lyomicron (Barcelona, Spain) and stored in a desiccator for posterior biochemical analysis.

2.6. Biochemical characterization

2.6.1. Protein content

The protein content was estimated by performing an elemental analysis (Vario EL III®, Elementar Analyzer System; GmbH, Hanau, Germany) according to the manufacturer's instructions and by multiplying the nitrogen content by a factor of 6.25 [41].

2.6.2. Pigments extraction and quantification

Chlorophyll extraction and quantification were performed according to Ritchie's method [42]. Briefly, 10 mg of biomass were weighed into a tube to which 2 g of glass beads ($d_p = 1$ mm) and 6 mL of acetone (99%)

were added. Milling was performed by vortexing for 10 min, followed by centrifugation for 10 min at 2547 *g* (Hermle® Z 300 centrifuge, Wehingen, Germany). The supernatant of the samples was collected and kept in the dark, while the extraction step was repeated until the pellet became colourless. Quantification of chlorophyll *a* and *b* was performed by measuring the absorbance of the supernatant at 630, 647, 664 and 691 nm, and applying Eqs. (6) and (7).

$$Chl_a = -0.3319Abs_{630} - 1.7485Abs_{647} + 11.9442Abs_{664} - 1.4306Abs_{691} \quad (6)$$

$$Chl_b = -1.2825Abs_{630} + 19.8839Abs_{647} - 4.8860Abs_{664} - 2.3416Abs_{691} \quad (7)$$

Carotenoid extraction was carried out with 1 mL of methanol containing 0.03% butylhydroxytoluene for 5–10 mg of dry biomass, followed by bead milling with 0.6 g of glass beads ($d_p \sim 425$–600 μm) using a mixer mill (Retsch MM 400) at 30 Hz for 3 min. Then, samples were centrifuged at 24,000 *g* for 3 min, and the supernatant was collected. The extraction procedure was repeated until both the pellet and the supernatant became colourless. Afterwards, the extracts were evaporated under continuous nitrogen flow. Finally, the dried samples were dissolved in 1 mL HPLC grade methanol and filtered through a 0.22 μm PTFE filter.

Carotenoids quantification was performed using a Chromaster HPLC System (Hitachi, VWR, Portugal), equipped with a diode array detector (5430 DAD, Hitachi, VWR, Portugal) and a Purospher® STAR RP-18 endcapped (Merck, Portugal) (250 × 2.1 mm, 5 μm) chromatographic column set at 20 °C. A mobile phase composed of solvent A (acetonitrile: water 9:1, v/v) and solvent B (ethyl acetate) were applied according to the following gradient: 0–16 min, 0–60% B; 16–30 min, 60% B; 30–32 min 100% B and 32–35 min 100% A [43]. A Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, US) was used for carotenoid identification. Quantification was carried out using calibration curves of neoxanthin, violaxanthin, lutein and β-carotene standards (Sigma-Aldrich, Portugal). The injection volume of both extracts and standards was 100 μL.

2.7. Statistical analysis

One-way ANOVA followed by Tukey HSD *post-hoc* multiple comparisons test at a probability level of 0.05 was performed using GraphPad Prism version 8.0.1 as well as graphical representation (GraphPad Software, San Diego, USA, http://www.graphpad/.com). For each test, the mean and standard deviation were determined among biological triplicates.

3. Results and discussion

3.1. EMS dose-response experiments

The first step of this work was to determine the survival rate of *S. rubescens* and *C. vulgaris* exposed to EMS (Fig. 2).

In *S. rubescens*, EMS concentrations of 100, 200, 300 and 400 mM led to the respective survival rates of 58%, 9%, 6% and 0.1%, while at higher concentrations of EMS the survival rate was 0% (Fig. 2). On the other hand, at 100 mM of EMS, the survival rate of *C. vulgaris* was 9%, which reduced to 2% at 150 mM and 0% for higher concentrations.

The optimal mutagen concentration to apply to a mutagenesis protocol should correspond to a survival rate between 5 and 10%, since lower survival rates tend to favour colonies with multiple mutations, and these should be avoided as they might compromise genome integrity and generate a more complex, pleiotropic phenotype that might revert more readily. On the other hand, survival rates should not be too high to improve the probability of finding cells with at least one mutation, decreasing the number of mutants to screen [13,44]. Accordingly,

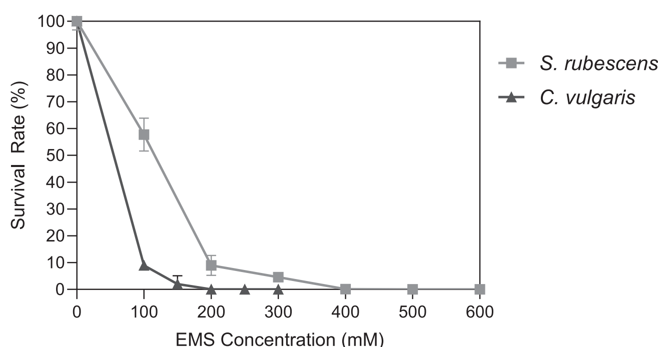

Fig. 2. Survival rate (%) of *S. rubescens* and *C. vulgaris* exposed to different ethyl methanesulfonate (EMS) concentrations. Results are shown as mean ± SD, $n = 3$.

the optimal EMS concentrations to use in the conditions tested were 200–300 mM and 100–150 mM for mutagenesis of *S. rubescens* and *C. vulgaris*, respectively.

Zhang et al. [45] studied the effect of EMS on *Scenedesmus* sp. (FACHB-489) and reported a survival rate of 46% at 200 mM EMS, 5-fold higher than the results obtained for *S. rubescens* strain in this experiment. On the other hand, varying susceptibilities of *Chlorella* sp. to EMS were reported by several authors [13,46–53]. Shin et al. [46] obtained a 10% survival rate with 150 mM EMS, while Guardini et al. [47] determined that 161 mM EMS resulted in a similar survival rate (5–10%). In contrast, Schüler et al. [13] described a 5–10% survival rate with 300 mM EMS.

The observed differing susceptibilities of *C. vulgaris* and *S. rubescens* to EMS are probably due to strain-specific differences in sensitivity to this agent and/or differences in the experimental conditions used. More specifically, it is noteworthy that, for example, in this work, 2.16×10^5 cells of *C. vulgaris* were subjected to EMS, which accounts for 14 times less cells than, for example the 3.20×10^6 cells reported in Schüler et al. [13], which resulted in a 50% lower EMS concentration needed to kill 90% of the cells under similar experimental conditions. Similarly, in this experiment, only 5–10% out of the 1.54×10^6 cells of *S. rubescens* survived 200 mM EMS, while Zhang et al. [45] reported 46% cell viability at the same EMS concentration, with 2.20×10^7 cells of *Scenedesmus* sp. In addition, these authors used different species, exposed the culture to light and used a different culture medium. Therefore, to decrease discrepancies in the results, reported protocols should be standardized, as the survival rate upon an EMS treatment can be influenced by factors such as species, strain, cell concentration, growth stage, and culture medium used [54,55]. Incubation conditions such as time, temperature and the presence or absence of light also significantly impact the results. In particular, exposure to light might increase the susceptibility of cells to the mutagenic agent and/or induce light-dependent DNA-repair mechanisms [54,55].

3.2. Oxyfluorfen toxicity

To determine the optimal range of oxyfluorfen for mutants' selection, several concentrations of this inhibitor were tested on *S. rubescens* (Fig. 3) and *C. vulgaris* (Fig. 4).

In 6-well plates, *S. rubescens* growth (Fig. 3) was fully inhibited by concentrations equal to or higher than 300 μg L^{-1} of oxyfluorfen, while only concentrations higher than 500 μg L^{-1} inhibited growth of *C. vulgaris* completely (Fig. 4).

As the optimal inhibitor concentration to use for mutant selection should be between the sublethal and lethal concentrations [21,48], the oxyfluorfen concentration ranges to test for *S. rubescens* and *C. vulgaris* strains after mutagenesis were established between 200 and 300 μg L^{-1} and 400–500 μg L^{-1}, respectively (Table S1 – Supplementary Material). This strategy ensures that the surviving colonies that gained a suitable

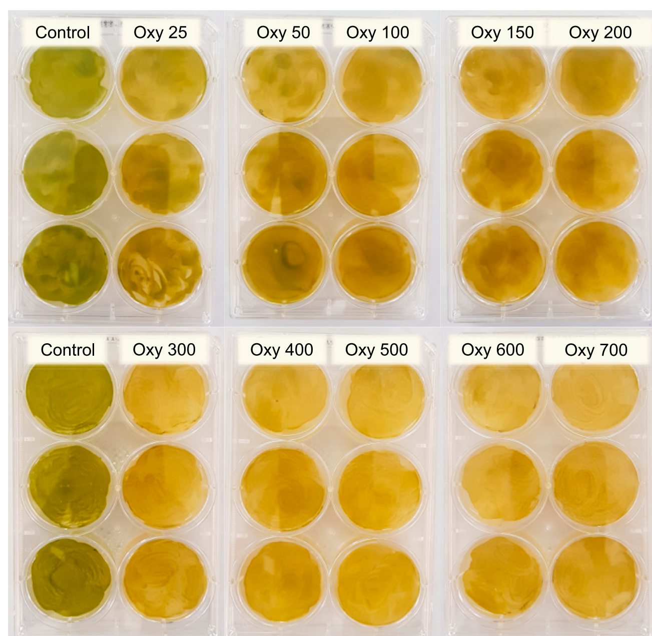

Fig. 3. *Scenedesmus rubescens* oxyfluorfen (Oxy) toxicity test. The culture in early exponential phase was plated onto PCA with Oxy and incubated in the dark. Oxyfluorfen concentrations are represented in μg L^{-1}.

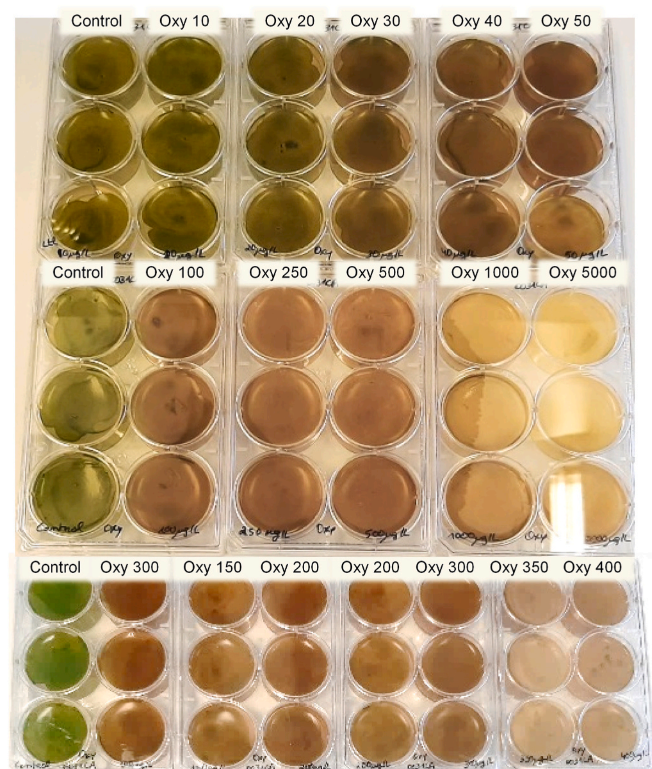

Fig. 4. *Chlorella vulgaris* oxyfluorfen (Oxy) toxicity test. The culture in early exponential phase was plated onto PCA with Oxy and incubated in the dark. Oxyfluorfen concentrations are represented in μg L^{-1}.

mutation that imparts their resistance to this chemical are more likely to be selected, avoiding non-mutant false positives and false negatives that have acquired a stable mutant phenotype.

In the presence of this inhibitor, both cultures acquired a brownish colour that can be associated with decreased chlorophyll contents and possibly with the toxic effect of increased oxidative stress that has been linked to diphenylethers, such as oxyfluorfen [33,56].

Geoffroy et al. [32] studied the toxicity of this inhibitor on the growth of an autotrophic culture of *Tetradesmus obliquus* (synonym of *Scenedesmus acutus*), in microplates with liquid medium. To inhibit the growth of 10% of the population (IC_{10}), 3 μg L^{-1} of oxyfluorfen was required, 15 μg L^{-1} for 50% (IC_{50}), while 22 μg L^{-1} were sufficient to inhibit the growth of 90% of the cells (IC_{90}). These concentrations are significantly lower than the ones reported in the present work. The difference in these values can be attributed to different experimental conditions that significantly impact the toxicity of oxyfluorfen such as the presence or absence of light, culture medium, cell concentration, well volume and respective altered partial pressure of atmospheric gases, temperature and agitation.

On the other hand, Cheng et al. [57] studied the toxicity of a similar diphenyl ether, acifluorfen, in *T. obliquus* when grown photoautotrophically in liquid medium at 25 °C, under continuous illumination on a 16:8 light:dark cycle. The effective concentration of acifluorfen resulting in a 50% decrease in *T. obliquus* growth (after 96 h) was 341 μg L^{-1}. Although this concentration is closer to the concentration of 300 μg L^{-1} of oxyfluorfen that inhibited growth of *S. rubescens*, it is noteworthy that the experimental conditions were also completely different.

Regarding *Chlorella* sp., Zhao et al. [58] studied the toxicity of a polybrominated diphenyl ether (BDE-47), a molecule with a chemical structure related to oxyfluorfen, and reported a 97% growth inhibition at a concentration of 120 μg L^{-1}. Once more, it is difficult to compare the inhibitory concentrations not only because different species and different inhibitors were used (although chemically related), but also due to several differences in the experimental conditions. However, this inhibitor seems to resemble oxyfluorfen with respect to the toxic effects caused on *Chlorella* sp. and *Scenedesmus* sp. [32,59].

Oxyfluorfen is a fluorinated chemical that induces a bleaching effect, i.e., decrease in pigments content, either by inhibiting the synthesis of these molecules and/or by inducing the loss of pigments. While inhibitors like norflurazon have been linked only to the first hypothesis, causing the accumulation of phytoene and other carotenoids precursors, oxyfluorfen also causes a loss of pigments in algae as aimed for this work [56]. As mentioned previously, oxyfluorfen has been reported to inhibit chlorophyll biosynthesis by inhibiting the enzyme PPO (Fig. 1), which leads to the accumulation of protoporphyrinogen IX [35,36,60]. This molecule goes through a non-enzymatic oxidation, causing the formation of ROS, which triggers lipid peroxidation. Since the precursor protoporphyrin IX is not produced, chlorophyll synthesis does not occur either. Moreover, phytotoxicity of these herbicides has been linked to the peroxidative degradation of cellular components, mainly membrane lipids [34]. In 1987, Lambert et al. [61] investigated the toxicity of this inhibitor to microalgae, namely the binding and peroxidative action of oxyfluorfen in *Scenedesmus acutus*. Geoffroy et al. [36] indicated antenna size and chlorophyll content as the most sensitive biomarkers to evaluate the toxic effect of oxyfluorfen on *T. obliquus*. More recently, the biochemical effects of oxyfluorfen on other species has also been reported [60,62].

Overall, oxyfluorfen affects the transcription levels and activities of enzymes of both carotenoids and chlorophyll biosynthetic pathways, which affect pigment biosynthesis, which in this study would lead to a loss of pigments and subsequent improved sensory properties of the microalgal biomass [33].

3.3. Isolation of mutants with different pigmentation

Based on the previous results, cells were mutagenized with EMS and exposed to oxyfluorfen. Mutagenized cultures of *S. rubescens* and *C. vulgaris* isolated on oxyfluorfen resulted in the appearance of yellow, brown and green colonies and also white in the case of *C. vulgaris* (Fig. 5).

A brownish *S. rubescens* colony isolated with 100 μg L^{-1} of

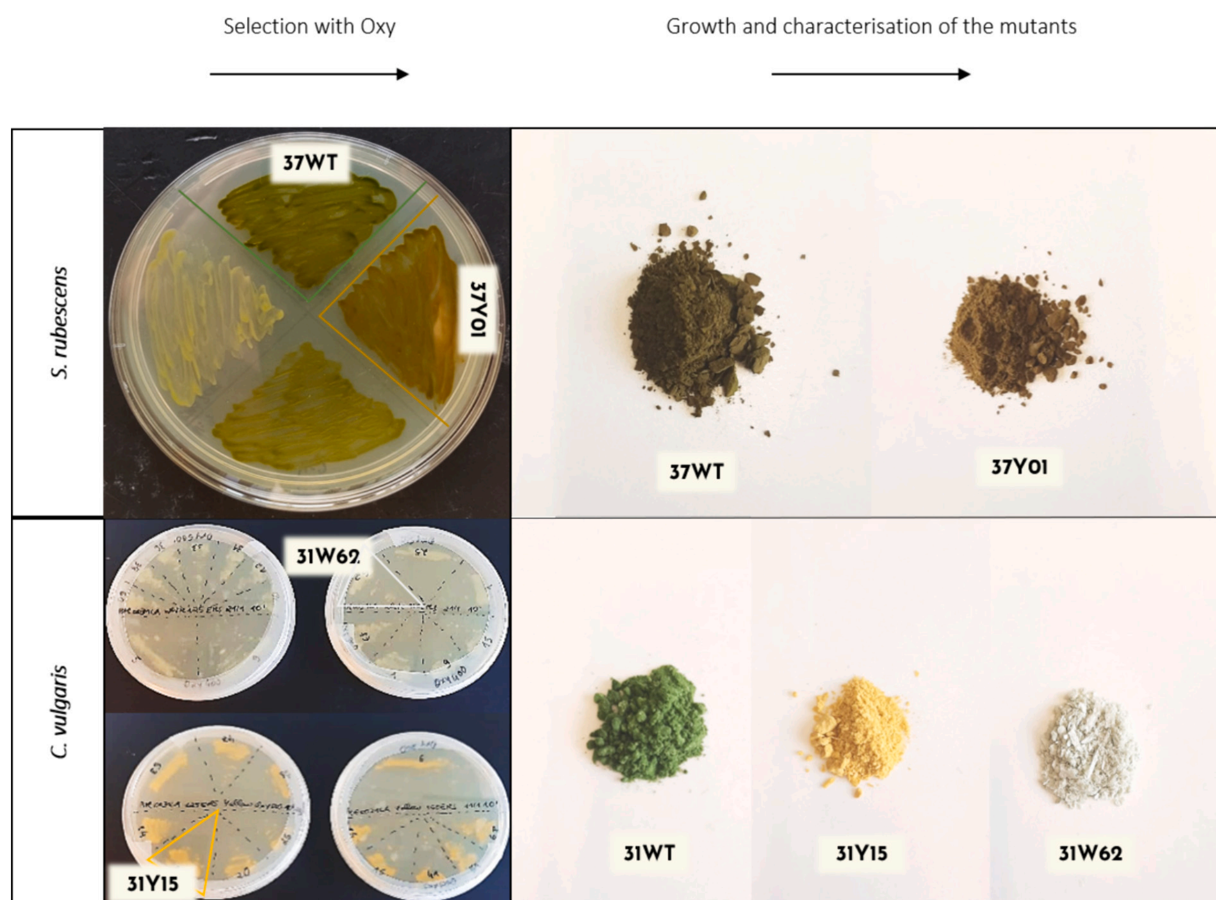

Fig. 5. Selection of mutants of *S. rubescens* and *C. vulgaris* with oxyfluorfen (oxy) and streaking on plate (left). Lyophilized biomass obtained after growth trials (right).

oxyfluorfen and generated with 200 mM of EMS, designated 37Y01, was picked and streaked for 10 generations to ensure phenotype stability (Table 1). Regarding *C. vulgaris*, 9 yellow and 11 white mutants were selected with this inhibitor (data not shown). Although the growth of all *C. vulgaris* mutants was compared among each other, only the most significant data for the continuity of this work are shown below, namely the characterization of the yellow and white mutants with growth performances comparable to the WT, that were selected for future work, 31Y15 and 31W62, both generated with 150 mM EMS and selected with 500 μg L^{-1} of oxyfluorfen (Table 1).

These results demonstrate the feasibility of using a metabolic inhibitor of the chlorophyll biosynthesis pathway, like oxyfluorfen, to select EMS-mutagenized cells with impaired pigmentation.

Random mutagenesis has been extensively used previously in several microalgal species, namely as a strategy to generate mutants with altered chlorophyll and carotenoid contents (Table S2 – Supplementary Material) [13,22,27,46–49,63–68]. Regarding Scenedesmaceae species, there are a few papers describing the application of EMS-based random mutagenesis. For example, Zhang et al. [45] used both EMS and UV-radiation to generate mutants of *Scenedesmus* sp. with a high biomass productivity, while Zhang et al. [69] isolated *Desmodesmus* sp. mutants not only with improved biomass yield but also lipid productivity. Conversely, *Chlorella* has been one of the most frequently targeted genera for strain improvement, accounting for 36% of the reports concerning microalgal random mutagenesis in 2022 [21].

Although with different goals, EMS-random mutagenesis has allowed to generate mutants with different pigmentation profiles, such as a pale-green phenotype, white and yellow strains and carotenoids hyper-producers [13,46–49,64,65]. The limiting step of this approach has always been keeping the number of mutants to be screened to a minimum and having a suitable screening strategy to identify and select cells exhibiting phenotypes of interest. Several screening strategies have been applied, such as the selection of colonies by visual appearance, more specifically by their colour [46,64,65]. To select randomly generated and differentially pigmented mutants more efficiently, high-throughput technologies as, for example, FACS, have been used [49,66,68]. On the other hand, metabolic inhibitors can be used to speed up the selection process. In particular, for the selection of mutants with different pigmentation, two inhibitors have been often reported: nicotine and norflurazon, which target the carotenoid pathway, leading to the generation of mutants with enhanced carotenoids content [21,27,47,63]. However, the isolation of mutants with norflurazon with decreased carotenoid and chlorophyll contents has also been reported [13,33]. Nevertheless, the application of a direct inhibitor of the chlorophyll biosynthetic pathway has not been reported, either to enhance chlorophyll content or to obtain chlorophyll-deficient mutants. This study describes for the first time the use of a chlorophyll biosynthetic pathway inhibitor, oxyfluorfen, that enabled the selection of chlorophyll-deficient mutants of *S. rubescens* and *C. vulgaris* with improved colour,

Table 1
Conditions applied in the isolation of *S. rubescens* and *C. vulgaris* mutants chosen for future characterization.

Species	Mutant	EMS concentration (mM)	Oxy concentration (μg L^{-1})
Scenedesmus rubescens	37Y01	200	100
Chlorella vulgaris	31Y15	150	500
Chlorella vulgaris	31W62	150	500

odour and taste. Moreover, the combined use of different selection strategies simultaneously might improve the success rate of random mutagenesis experiments [19,21,70]. For example, Yi et al. [22] combined the use of diphenylamine, another inhibitor of the carotenoid biosynthetic pathway, with the selection of colonies by visual appearance (size and colour), with a subsequent FACS selection step. This strategy led to a 69% increment of fucoxanthin content in a *Phaeodactylum tricornutum* mutant compared to the levels found in the WT.

3.4. Wildtype vs mutants' growth performances

Oxyfluorfen-derived *S. rubescens* and *C. vulgaris* mutants and respective wildtype strains were compared at lab-scale regarding their growth performance, namely biomass productivity (P) and growth rate (μ) (Fig. 6).

As for *S. rubescens*, the WT productivity and growth rate (2.13 ± 0.08 g L^{-1} day^{-1} and 1.17 ± 0.03 day^{-1}) were significantly higher than those of 37Y01 (1.66 ± 0.10 g L^{-1} day^{-1} and 1.05 ± 0.02 day^{-1}).

Chlorella vulgaris WT strain showed significantly higher biomass productivity and growth rate values than those of 37WT, 2.39 ± 0.01 g L^{-1} day^{-1} and 1.31 ± 0.01 day^{-1}, respectively ($p < 0.05$). The yellow mutant 31Y15 displayed a biomass productivity of 2.19 ± 0.15 g L^{-1} day^{-1}), with no significant differences compared to the WT ($p > 0.05$). However, the growth rate of 31Y15 (0.99 ± 0.02 day^{-1}) was significantly lower than that of the WT ($p < 0.05$). The white mutant 31W62 displayed a significantly lower biomass productivity (1.84 ± 0.08 g L^{-1} day^{-1}) and growth rate (0.93 ± 0.01 g L^{-1} day^{-1}) compared to the WT and 31Y15 (Fig. 6) ($p < 0.05$).

Chlorella and *Scenedesmus* have been reported to achieve some of the highest biomass and protein productivities [13,39,40,71], which might be related to the fact that these are two of the few genera of microalgae with the ability to grow heterotrophically, which allows to achieve much higher cell concentrations than with photoautotrophic cultivation [8,9,39,40].

Recently, Espírito Santo et al. [39] studied the heterotrophic growth of the same wildtype strain of *S. rubescens* in similar conditions, reaching a biomass productivity of 2.86 g L^{-1} day^{-1} and a specific growth rate of 1.18 day^{-1}. Although the productivity was higher than the one obtained in the present work, the growth rate was similar. Other studies on the heterotrophic growth of *Scenedesmus* spp. have, however, attained lower values, demonstrating the variability of growth within microalgae of the same genus and under different cultivation conditions. For example, Ren et al. [72] studied the heterotrophic growth of *Scenedesmus* sp. and reached a lower growth rate of 0.82 day^{-1}, while for *T. obliquus* a biomass productivity of 0.46 g L^{-1} day^{-1} was reported [73].

Regarding *C. vulgaris* growth performance, there is a wide range of values that can be found in literature, which is always dependent on cultivation conditions, medium used, type of reactor and scale. Under lab-scale heterotrophic conditions, most studies report growth rates and biomass productivities lower than the ones reported in the present work, between 0.55 and 0.79 day^{-1} and 1.65–1.99 g L^{-1} day^{-1} [74–77].

Among several reports in literature, different outcomes in terms of biomass productivity and growth rate have been reported upon mutagenesis. Similarly to the results obtained for the WT and the 31Y15 yellow mutant strain, which displayed similar growth performances, Schüler et al. [13] reported a statistically equivalent growth for the WT and a yellow mutant in an Erlenmeyer assay under similar conditions. Moreover, the same authors also generated a white mutant that propagated slower than the WT and the yellow mutant, similarly to what is described in the present study. Concerning the improvement of biomass productivity through random mutagenesis, there are several reports on *C. vulgaris* [49,51], *Chlorella* spp. [52,78] and other species [79]. Aiming at improving photosynthetic efficiency, Patil et al. [64] developed chlorophyll-deficient mutants of *C. vulgaris* using EMS-dependent mutagenesis, which allowed the biomass productivity to increase by 27% as compared to that of the WT, while Shin et al. [46] reported a 45% increment under autotrophic conditions.

Regarding Scenedesmeceae genus, growth has also been previously improved by resorting to random mutagenesis. For example, Xi et al. [80] isolated a *T. obliquus* mutant through $^{12}C^{6+}$ ion beam mutagenesis, which resulted in the generation of a mutant with a 57% and 25% improvement in biomass productivity and growth rate, respectively.

Overall, many of these reports in which biomass productivities and growth rates were improved, described decreased chlorophyll levels along with smaller antenna sizes, which under autotrophic cultivation conditions will likely increase the efficiency of the process by preventing self-shading effects and limited growth [21]. The same seems inapplicable to heterotrophic cultivation, so that mutagenesis along with impaired pigmentation likely leads to slower or similar growth performances comparing to the wildtype strains. Moreover, in this work the growth parameters obtained are the same or close to the maximum reported for these species at this scale. Finally, higher biomass productivities and growth rates have been reported upon scale-up and process optimization, both for *S. rubescens* [39] and *C. vulgaris* [40], which points out the potential of further improving the mutants selected in this work.

Strain	P (g L^{-1} d^{-1})	μ (d^{-1})
37WT	2.13 ± 0.08^{b}	1.17 ± 0.03^{b}
37Y01	1.66 ± 0.10^{c}	1.05 ± 0.02^{c}
31WT	2.39 ± 0.01^{a}	1.31 ± 0.01^{a}
31Y15	2.19 ± 0.15^{ab}	0.99 ± 0.02^{d}
31W62	1.84 ± 0.08^{c}	0.93 ± 0.01^{e}

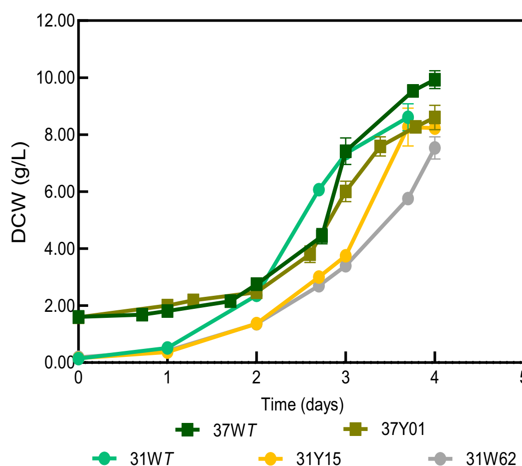

Fig. 6. Biomass productivies (P) and growth rates (μ) (on the left) and growth curves (on the right) of *S. rubescens* and *C. vulgaris* WT and mutant strains, grown heterotrophically in 250-mL Erlenmeyer flasks for 4 days (in the absence of inhibitor). Results are shown as mean ± SD, $n = 3$. Different letters indicate significant differences ($p < 0.05$) between strains.

3.5. Biochemical characterization

3.5.1. Protein content and productivity

The protein content and productivity of both wildtype strains of *S. rubescens* and *C. vulgaris* and respective mutants are presented in the figure below (Fig. 7).

S. rubescens WT displayed a significantly higher protein content, 25.0% of DW, compared to that of mutant 37Y01, 19.1% ($p < 0.05$), with protein productivities of 2.1 and 1.7 g L^{-1} d^{-1}, respectively (Fig. 7). Concerning *C. vulgaris*, the ranges of protein contents and productivities obtained were between 42.8 and 44.3% and 1.8–2.4 g L^{-1} d^{-1}, respectively (Fig. 7), whose contents were considerably higher than the values of *S. rubescens* ($p < 0.05$). The yellow (31Y15) and white (31W62) mutants selected exhibited protein contents of 44.25% and 42.80%, which were similar to the one of the WT strain containing 43.28% (Fig. 7).

A recent study reported a higher protein content of 31–33% [39], for the same *S. rubescens* strain used, grown in the same culture medium, but in a fed-batch 7-L bench top fermenter (Table 2). Furthermore, when grown photoautotrophically, this strain can reach protein contents as high as 60% (Table 2) [71]. Other studies carried out with Scenedesmeceae report a wider range, 13–54%, when grown autotrophically (Table 2). The species *T. obliquus* was also reported to have a protein content of 44%, when grown autotrophically [81]. Under heterotrophic conditions, it has been suggested that *T. obliquus* can similarly achieve a protein content of 55% [82].

As for the trophic mode, there is a wide range of values that can be found for *C. vulgaris* protein content (Table 2). Under autotrophic cultivation conditions, Chen et al. [83] achieved a protein content of 52.3% DW, while Lai et al. [84] reported half of that, reaching only 24.9% DW. However, under heterotrophic conditions and with microalgae of the same species, both Lau et al. [75] and Barros et al. [40] attained lower protein levels, 20.0%, even though more recently Xie et al. [85] and Cai et al. [77] reported values 2- and 3-fold higher, reaching 44.3% and 64.1% DW, respectively. Interestingly, Barros et al. [40] reported the use of a combination of the two trophic modes as a strategy to achieve the highest biomass and protein productivities possible within the shortest time period, which enabled a protein content of 52.2% DW.

Although protein content improvement was not the goal of this study neither a strain selection criterion, there are almost no reports regarding the improvement of protein contents on microalgae through random mutagenesis, since most studies found in literature are more directed towards lipids and carbohydrates. Nonetheless, Schüler et al. [13] reported increased protein content in a WT *C. vulgaris* strain (30.5%) in its yellow (39.5%) and white (48.7%) mutants. In the present study, as mentioned above, there were no significant differences among the protein contents of the WT and the yellow and white mutants isolated. However, the protein content reported for the WT is already very similar to the highest value reported in Schüler et al. [13] and close to the highest values found in literature (40–60%) (Table 2). It becomes clear that there is a significant heterogeneity of values regarding protein content of these species (Table 2), which might be explained by diverse reasons, namely trophic mode, cultivation conditions, strain used, culture medium, the method used to quantify protein content, among others. Moreover, the nitrogen concentration during growth is a crucial factor for protein synthesis. In fact, it has been shown that the protein content of *T. obliquus* can range from 11.4 to 55.6% if the nitrogen levels in the medium are increased [82].

Taken together, these results confirm that *C. vulgaris* is one of the most promising microalgal species as a protein source [83] and the contents of the strains generated can probably be further enhanced by optimization of cultivation conditions. Although several biochemical characterizations of both *Chlorella* and *Scenedesmus* biomasses have been

Table 2
Reports of protein content of *C. vulgaris*, *S. rubescens* and *Scenedesmus* spp. under different trophic modes. Examples in heterotrophic conditions, are highlighted in bold in the trophic mode column.

Species	Protein content (% DW)	Trophic mode	Reference
Scenedesmus bajacalifornicus	32.9	Autotrophic	[86]
Scenedesmus rubescens	31.0–33.0	**Heterotrophic**	[39]
Scenedesmus rubescens	60.0	Autotrophic	[71]
Scenedesmus sp.	56.0	Autotrophic	[87]
Scenedesmus sp.	12.5–28.2	Autotrophic	[88]
Tetradesmus obliquus	28.5–44.4	Autotrophic	[81]
Tetradesmus obliquus	55.0	**Heterotrophic**	[82]
Scenedesmus rubescens			
37WT	25.0 ± 1.2	**Heterotrophic**	This study
37Y01	19.1 ± 0.6		
Chlorella vulgaris			
WT	30.5		
MT01	39.5	**Heterotrophic**	[13]
MT02	48.7		
Chlorella vulgaris	52.2	Two-stage	[40]
Chlorella vulgaris	20.0	**Heterotrophic**	[75]
Chlorella vulgaris	64.1	**Heterotrophic**	[77]
Chlorella vulgaris	52.3	Autotrophic	[83]
Chlorella vulgaris	24.9	Autotrophic	[84]
Chlorella vulgaris	44.3	**Heterotrophic**	[85]
Chlorella vulgaris			
31WT	43.3 ± 0.5		
31Y15	44.3 ± 0.7	**Heterotrophic**	This study
31W62	42.8 ± 1.1		

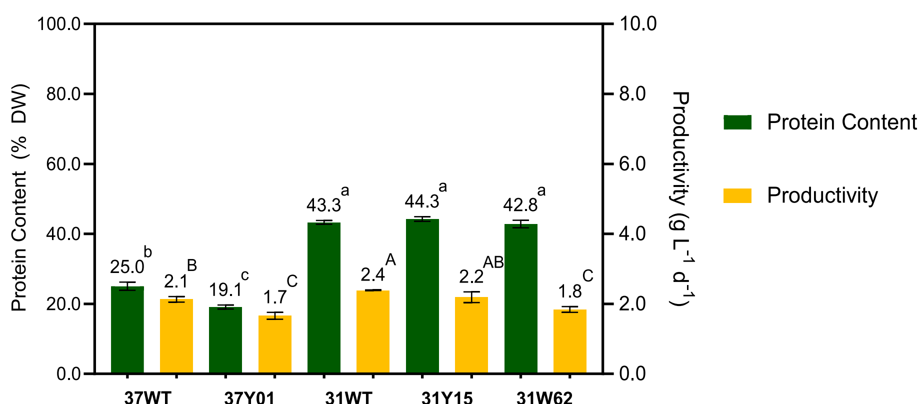

Fig. 7. Protein contents (% of DW) and protein productivities (g L^{-1} d^{-1}) of *S. rubescens* and *C. vulgaris* wildtype and mutant strains. Protein contents were determined by measuring total N in an elemental analyzer. Results are shown as mean ± SD, $n = 3$. Different letters indicate significant differences ($p < 0.05$) between strains.

reported, namely regarding proximal composition [13,39] and amino acids profiles [89,90], it would be of great interest to analyse and compare the nutritional profiles of wildtype strains and mutants, namely mutants selected with different inhibitors. Few studies have been carried out regarding this matter. For example, Maurício et al. [91] compared the lipid profile of *Chlorella vulgaris* wildtype and yellow and white mutants, while Cabrol et al. [92] compared the amino acids profiles obtained in pork frankfurters with and without enrichment with the same yellow and white mutants.

As for future perspectives concerning the improvement of protein productivity, it is key to study the mechanisms and metabolic pathways underlying the production and accumulation of protein in the microalgal cell. In addition, screening methods must be developed to allow the selection of mutants with even higher protein contents, if possible.

3.5.2. Chlorophyll content and carotenoids profile

Carotenoids and chlorophyll concentrations of both WTs and respective mutants were analysed. The profiles of pigments are shown in Table 3.

Macroscopically, 37WT colonies were dark-green, while 37Y01 presented a brownish colour. This difference was apparently due to their pigmentation profiles. Accordingly, mutant 37Y01 displayed a 55% and a 18% decrease in total chlorophyll and carotenoid contents, respectively, compared to the WT. Despite the decrease in the carotenoid content, neoxanthin was detected in 37Y01 biomass at a concentration of 0.31 mg g^{-1}, whereas this carotenoid was not present in the WT profile (Table 3). On the other hand, the *β*-carotene content in the mutant strain decreased by 68%, from 0.88 mg g^{-1} DW, to 0.28 mg g^{-1} DW (Table 3).

C. vulgaris WT biomass also presented a dark-green colour, while mutant 31Y15 and 31W62 exhibited a yellow and white colour, respectively. Pigments profile of the three strains reflected these descriptions (Table 3). As expected, the total chlorophyll content of the WT was the highest, 6.7 mg g^{-1} of DW, and was practically null for both mutants (~95% reduction), with about 0.3 mg g^{-1} of DW for both the yellow and white mutant. The WT displayed high concentrations of carotenoids, namely 0.27, 0.52 and 1.29 mg g^{-1} of DW for neoxanthin, lutein and *β*-carotene, respectively. However, none of the carotenoids analysed were identified in the biomass of the yellow and white mutants.

Mutants with different pigments' profile have been selected after mutagenesis whether aiming at higher chlorophyll contents or lower, depending on the authors' intent (Table 4 and Table S2 - Supplementary Materials). A chlorophyll-deficient mutant of *Desmodesmus armatus* has been previously isolated by resorting to UV-mutagenesis, although the objective was to isolate mutants resistant to fluazinam, which resulted in increased autotrophic growth rate and biomass productivity [93]. On the contrary, the mutant of *T. obliquus* isolated by Xi et al. [80], with the objective of obtaining increased photosynthetic efficiency and lipid content, displayed a 33% increment in chlorophyll content and a 48% increase in total carotenoids content. There are also some reports of pale-green and/or chlorophyll-deficient phenotypes of *C. vulgaris*, selected with the purpose of having decreased antenna sizes and improved photosynthetic efficiencies (Table 4). For example, Shin et al. [46] and Dall'Osto et al. [48] obtained a 50% chlorophyll reduction through EMS-mutagenesis and selection of mutants with lighter green colour, while Cazzaniga et al. [68] achieved the same 50% reduction but with UV-mutagenesis and selection through chlorophyll fluorescence. Moreover, Shin et al. [46] also reported a reduction of 75% of the carotenoids content, while Cazzaniga et al. [68] reported lower levels of neoxanthin and lutein for the mutants, while *β*-carotene, violaxanthin and zeaxanthin were more abundant, comparing to the WT.

According to several published data, differently coloured chlorophyll-deficient mutants (e.g., yellow and white) in heterotrophic conditions, as in the present work, generally exhibit lower chlorophyll and carotenoids contents. Schüler et al. [13] isolated a yellow and white heterotrophic mutants of *C. vulgaris*, selected with norflurazon and by colour, that contained less 82% and 95% chlorophyll than the WT, respectively. Additionally, the carotenoids content of both mutants was much lower than the WT, being below the limit of quantification (LOQ) for the white one, while the most abundant carotenoid in the yellow mutant was lutein with 0.86 mg g^{-1} of DW. The lutein content of the WT in the present work was lower and not even detectable for the yellow and white mutants. However, for example *β*-carotene was much higher for this WT (1.29 mg g^{-1}), while in Schüler et al. [13] the WT had only 0.28 mg g^{-1} as well as neoxanthin and violaxanthin. These differences can be related to the use of different strains, different cultivation conditions, namely culture medium, and also to different extraction and quantification methods, such as instrumental techniques used (spectrophotometric or chromatographic).

In contrast to the objective of the present study, there are also several works published targeting the increment of carotenoids content, through mutagenesis and different selection strategies (Table 4 and Table S2 - Supplementary Materials). Recently, Eregie et al. [94] selected larger colonies after UV-mutagenesis, which led to a 1.2-fold increase of the total carotenoid content of *S. vacuolatus*. Concerning lutein, one of the most abundant carotenoids in *Chlorella* sp., Cordero et al. [63] enhanced this pigment by 2-fold in an autotrophic *Chlorella sorokiniana*, after mutagenesis with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and selection with nicotine, while Chen et al. [27] attained a lutein productivity 1.5-fold higher than the WT, but in mixotrophic conditions. On the other hand, Huang et al. [67] attained some of the highest carotenoid concentrations reported for this genus by inducing the accumulation of lutein, β-carotene and zeaxanthin on *Chlorella zofingiensis*, after mutagenesis with MNNG, followed by selection under dim light and diphenylamine, attaining 13.8 mg g^{-1}, 7.18 mg g^{-1} and 7 mg g^{-1}, an increment of 2-, 5- and 7-fold of lutein, β-carotene and zeaxanthin, respectively.

Oxyfluorfen affects the transcription levels and activities of enzymes of both carotenoids and chlorophyll biosynthetic pathways, which affect pigment biosynthesis. Altered metabolite levels on one pathway impact the gene expression in the other pathway, which also varies with the level of oxidative stress that cells are exposed to and often cause unpredictable effects on pigment biosynthesis. As discussed above, carotenoid inhibitors, as norflurazon or nicotine, sometimes lead to the accumulation of some carotenoids [47,63], but it has also been reported that it might lead to decreased carotenoid concentrations [13,33]. Accordingly, the exposure to these inhibitors can either induce the accumulation of chlorophyll or cause its depletion. Likewise, it is not straightforward whether oxyfluorfen will cause chlorophyll depletion or accumulation as well as its effect on the biosynthesis of carotenoids,

Table 3
Carotenoids concentrations (mg g^{-1} of DW) of wildtypes and mutants of *S. rubescens* and *C. vulgaris*, determined by HPLC, and chlorophyll contents (mg g^{-1} of DW) determined by Ritchie method [42]. n.d. – not detected.

Species	Strain	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Neoxanthin	Lutein	*β*-Carotene
S. rubescens	37WT	16.45 ± 1.12	6.58 ± 0.94	23.07 ± 2.06	n.d.	0.53 ± 0.02	0.88 ± 0.04
S. rubescens	37Y01	6.23 ± 0.25	4.09 ± 0.39	10.32 ± 0.63	0.31 ± 0.05	0.50 ± 0.02	0.28 ± 0.03
C. vulgaris	31WT	4.79 ± 0.29	1.89 ± 0.29	6.68 ± 0.57	0.27 ± 0.02	0.52 ± 0.01	1.29 ± 0.10
C. vulgaris	31Y15	0.14 ± 0.03	0.18 ± 0.03	0.32 ± 0.05	n.d.	n.d.	n.d.
C. vulgaris	31 W62	0.12 ± 0.03	0.14 ± 0.03	0.26 ± 0.06	n.d.	n.d.	n.d.

Table 4
Examples of random mutagenesis reports that generated *C. vulgaris* and *S. rubescens* mutants with altered chlorophyll and carotenoid contents. An extended version of this table can be found in the Supplementary Materials (Table S2).

Species	Mutagenic agent	Target	Screening	Improvement	Reference
C. vulgaris	EMS	Chlorophyll deficiency	Colour and Norflurazon	Up to 99% decreased chlorophyll and 60% increased protein content.	[13]
C. vulgaris	EMS	Chlorophyll deficiency	Colour	Reduction by 57% in chlorophyll *a*, 76% in chlorophyll *b* and 45% increase in biomass productivity.	[46]
C. vulgaris	EMS	Chlorophyll deficiency; oxidative stress resistance	Colour; Red Bengal 12 μM	50% chlorophyll reduction; 68% higher biomass yield.	[48]
D. armatus	UV	Fluazinam tolerance	Fluazinam	33–38% decrease in chlorophyll fluorescence; improved productivity and quantum efficiency.	[93]
S. vacuolatus	UV	Improved biodegradative performance	Chlorophyll content; Substrate affinity; Sugar utilization; Growth rate	89% increase in chlorophyll *a* content, 24% carotenoid content and 44% in protein content.	[94]
T. obliquus	$^{12}C^{6+}$ ion beam	Increased photosynthetic efficiency; Increased lipid content	Chlorophyll fluorescence	Up to 48% increase in carotenoid content; up to 33% in chlorophyll *a* content; improved photosynthetic efficiency and lipid productivity; decreased protein content.	[80]
S. rubescens and *C. vulgaris*	EMS	Chlorophyll deficiency	Oxyfluorfen	55% and 95% chlorophyll reduction.	This study

which will also be influenced by the cultivation conditions. In the future it would be of great interest to study which genes were mutated through the usage of this inhibitor to understand the impact on the pigments' metabolic pathways of these mutated cells.

4. Conclusions

The metabolic inhibitor oxyfluorfen, tested for the first time as a selection strategy upon mutagenesis, allowed the isolation of chlorophyll-deficient mutants of *S. rubescens* and *C. vulgaris* with a 55% and 95% decrease in total chlorophyll content, respectively. *S. rubescens* WT and mutant 37Y01 exhibited protein contents between 19.1 and 25.0% DW, while *C. vulgaris* WT, yellow 31Y15, and white 31W62 mutants reached higher contents, 42.8–44.3% DW. Therefore, *C. vulgaris* mutant strains are more promising protein sources for developing novel food and feed applications with more appealing organoleptic characteristics due to the high protein and almost null chlorophyll contents. Nevertheless, their nutritional profile should be further characterised since it might present other interesting features, such as amino acid and lipids profile, vitamins, minerals and other bioactive compounds.

Overall, the oxyfluorfen-based random mutagenesis pipeline reported here established stable chlorophyll-deficient mutants of *C. vulgaris* and *S. rubescens* in an efficient, straightforward manner, expanding the mutant generation/selection pipeline as well as the portfolio of strains with high potential for nutritional applications.

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CRediT authorship contribution statement

Mafalda Trovão: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Lucas Cardoso:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Lisa Schüler:** Writing – review & editing, Supervision, Software, Project administration, Methodology, Investigation. **Adriana Machado:** Methodology, Investigation, Formal analysis, Conceptualization. **Gonçalo Espírito Santo:** Methodology, Investigation, Formal analysis. **Humberto Pedroso:** Methodology, Investigation, Formal analysis. **Ana Reis:** Methodology, Investigation, Formal analysis. **Ana Barros:** Writing – review & editing, Supervision, Methodology. **Nádia Correia:** Methodology, Investigation, Formal analysis. **Monya Costa:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Sara Ferreira:** Supervision, Methodology, Investigation, Formal analysis. **Helena Cardoso:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marília Mateus:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Joana Silva:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Hugo Pereira:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Filomena Freitas:** Writing – review & editing, Supervision, Resources, Funding acquisition. **João Varela:** Writing – review & editing, Supervision, Software, Project administration, Methodology.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103572.

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