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# *Effect of Fluazifop-P-butyl herbicide on carbohydrate, protein, and fatty acid compositions of Auxenochlorella pyrenoidosa and Raphidocelis subcapitata*

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**ABSTRACT:** Herbicides have been a major issue in terms of environmental and human safety; thus, a greater interpretation of the influence of this herbicide on the aquatic environment is necessary. In this study *Auxenochlorella pyrenoidosa* is treated with two sublethal concentrations from each form of fluazifop-P-butyl, 0.237 and 1.09 mg/L from traditional fluazifop-P-butyl (TFL) and 0.357 & 1.43 mg/L from nano fluazifop-P-butyl (NFL). Also, *Raphidocelis subcapitata* is treated with 0.029 & 0.117 mg/L from TFL and 0.119 & 0.476 mg/L from NFL. Total carbohydrate, protein, lipid contents and fatty acid compositions in both microalgal cells were evaluated after exposure time (96 h). Results showed that treated cells of *R. subcapitata* with 0.117 mg/L of TFL increased carbohydrate content by 78.87% when compared to the control. Treated cells of *A. pyrenoidosa* with 1.43 mg/L of NFL showed the highest decrease in total protein content (76.04%) as compared to the control. Total lipids increased by 136.22% in cells of *A. pyrenoidosa* treated with 1.09 mg/L of TFL compared to the control. Treated cells of *A. pyrenoidosa* with 0.273 mg/L of TFL showed an increase of total saturated and total unsaturated fatty acids of 118.71% and 186.85%, respectively, compared with the control. Overall, this study shows that TFL and NFL herbicide impacted on some biochemical components of the two studied algae, thereby altering the nutritional quality of this resource for primary consumers. Also, this study may enhance the possibility of using fatty acids as biomarkers of herbicide exposure assessment in freshwater ecosystems.

**Keywords**: Herbicide- Nano-emulsion- Microalgae- Toxicity- Fatty acids

## INTRODUCTION

The rising application of pesticides, particularly herbicides, in modern agriculture has generated significant apprehension regarding their potential detrimental effects on aquatic ecosystems (Narayanan *et al*., 2024). However, studies published after the International Agency for Research on Cancer (IARC) monograph indicate that chemicals from all major pesticide categories (such as insecticides, herbicides, fungicides, and fumigants) are linked to human cancer (Alavanja *et al*., 2013).

Meanwhile, paraquat (herbicide) is a major cause of fatal poisoning in many regions, particularly in agricultural nations. Its extreme toxicity, even in small doses, leads to swift damage to several organs, particularly the kidneys, lungs, and liver, primarily due to injury caused by free radicals (Asaduzzaman *et al*., 2023). Herbicides can permeate aquatic environments through spray drift, soil leaching, surface runoff, and erosion, thus contaminating water bodies and potentially inflicting harmful effects on non-target organisms (Jiao *et al*., 2022). Additionally, nano-pesticides can enter the surface of water bodies and degrade their quality, which might have a negative effect on ecosystems (Ale *et al*., 2023).

Fluazifop-P-butyl (FL) which IUPAC (International Union of Pure and Applied Chemistry) Name is Butyl (R)-2-[4-(5-trifluoromethyl-2-pyridyloxy) phenoxy] propionate has been classified as a post-emergence phenoxy herbicide according to Ragab *et al*. (2023). The function of this herbicide is to manage annual and perennial grasses after they emerge (Blake *et al*., 2012). It performs its function through the inhibition of the acetyl-CoA carboxylase enzyme that produces malonyl-CoA through lipid metabolism (Horbowicz *et al*., 2013). According to Ore and Olayinka, (2017) fluazifop-p-butyl is rapidly absorbed via leaf surfaces and transformed into fluazifop-p, mostly fluazifop acid, and additional minor metabolites. Currently, there are critical information gaps for some herbicide actives, such as fluazifop-P-butyl, that have only been explored as commercial formulations containing a range of unknown compounds (Dennis *et al*., 2023).

The evaluated drinking water concentrations of fluazifop-P-butyl for acute exposures to ground water and surface water were 6.8 ppb and 56.6 ppb, respectively, while for chronic exposure they were 3.39 ppb for ground water and 4.41 ppb for surface water (EPA, 2017). On the other hand, there are previous studies about the effect of fluazifop-P-butyl on fish. For instance, the acute toxic lethal concentration ($LC_{50}$) of fluazifop-P-butyl was estimated to be 1.94 mg/L for *Oreochromis niloticus* (Nile tilapia) larvae after 24 h exposure (Cansev and Münir, 2015). In addition, the $LC_{50}$ of this herbicide for

*Clarias gariepinus* (African sharptooth catfish) was estimated to be 17.17 mg/L after 96 h of exposure (Ragab *et al*., 2023).

Abdollahdokht *et al*. (2022) assessed the application of nanotechnology in pesticides, including electrospun nanofibers, nanogels, nano-emulsions, and nano-encapsulates. For instance, surfactants are exploited to form nano-emulsions, which are biphasic dispersion systems. These nano-emulsions are highly beneficial due to their kinetic stability and ability to degrade dimly water-soluble herbicides into minute oil droplets, boosting their efficiency and bioavailability which makes these nano-emulsions extremely advantageous (Ale *et al*., 2023).

In various studies, traditional herbicides have demonstrated inconsistent release kinetics and poor water solubility. It encourages farmers to use more pesticides in agricultural fields, which raises application costs, causes herbicide resistance, and accumulates toxins in the environment. On the other hand, nano herbicides minimize labor expenses for applying a smaller amount of herbicide and dismiss environmental concerns (An *et al*., 2022).

By changing the species composition of an algal community, herbicides can affect the structure and function of aquatic ecosystems (Smythers *et al*., 2019). To resist the negative effects of certain pesticides, algae depend on metabolism as a critical defensive mechanism (Narayanan *et al*., 2024). As one of the primary producers, green algae are an important component of the aquatic ecosystem. Considering their extreme reactivity to harmful substances in the water, they are commonly employed to evaluate the quality of the aquatic environment (Wang *et al*., 2019). *Auxenochlorella pyrenoidosa*, being among ubiquitous unicellular green microalgae, has been recognized for its exceptional photosynthetic effectiveness. Because of its susceptibility to toxins and rapid reproduction, it has been used as a model of biology for assessing contaminants in the environment (Duan *et al*., 2019; Feng *et al*., 2022). Moreover, the unicellular green unicellular microalga *Raphidocelis subcapitata* is recognized for its remarkable sensitivity to a diverse scale of chemicals. Consequently, it has been authorized for ecotoxicity assessments by several international agencies (Machado and Soares, 2024).

This work aims to assess the effects of two sublethal concentrations from two forms of Fluazifop-P-butyl (FL) herbicide (traditional and nano) on the compositions of carbohydrate, protein, lipid, and fatty acid in the freshwater microalgae *R*. *subcapitata* and *A*. *pyrenoidosa*. The two forms were used in this study to evaluate the efficiency of their effect on the studied algae as two model cells. Notably, this work is the first to look at how the biochemical components of the studied microalgae are affected by FL in both its traditional and nano-forms.

## MATERIALS AND METHODS

### Microalgae and growth conditions

This study used the unicellular green freshwater *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen, and O. M. Skulberg, as well as *Auxenochlorella pyrenoidosa* (H. Chick) Molinari & Calvo-Pérez. *Raphidocelis subcapitata* was gotten from the phycology laboratory at the Department of Botany, Faculty of Science, Mansoura University, Egypt. *Auxenochlorella pyrenoidosa* was gotten from the National Institute of Oceanography and Fisheries, Alexandria, Egypt. We used Allen and Arnon's medium (Miller *et al*., 1979) to culture both microalgae at 25 ± 3°C with continuous "Cool-White" fluorescent lighting (195.08 µmol photons $m^{-2}$. $s^{-1}$) and pH (6.8).

### Synthesis of nano-emulsions of herbicide

Shoura for Chemicals Co., Egypt, provided fluazifop-P-butyl (FL) (High class penta® 15% EC). On the other hand, the Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center (ARC), Cairo, Egypt supplied the active ingredient of FL (purity 90%) that exploited in the procedure of forming the nano-emulsions. The quantification of FL was adjusted in this Institute (CAPL, ARC) for active ingredient percentage in the traditional before certification for pesticide registration following by marketing procedures. An ultrasonic probe (SONIC; Vibra - Cell™, Church Hill Rd., Newtown, CT, USA) at 60 $H_Z$ for 10 minutes and a rest time of 30 seconds for each cycle underneath cooling has been used for the preparation of a nano-emulsion of FL (5%) underneath high energy mode. At a ratio of 1:20 v/v, the AI (5 g) of FL was dissolved in 5 mL of vegetable oil and then added to the liquid phase (95 mL of water). The main surfactant (Tween 80) dropped into the mixture at percentage 5% of the total volume after justification by measuring the surface tension value at converted point of surfactant in water. Therefore, to improve the hydrophobic/lipophilic balance (HLB), 0.05% of polymer (polyvinyl pyriddinol, PVP) was added according to the method described by Abdel-Halim *et al*. (2021)

**Sublethal treatments**

In this study, *A. pyrenoidosa* is treated with two sublethal concentrations from each form of herbicide, 0.237 & 1.09 mg/L from TFL and 0.357 & 1.43 mg/L from NFL. Also, *R. subcapitata* is treated with 0.029 & 0.117 mg/L from TFL and 0.119 & 0.476 mg/L from NFL herbicide. These treatments were compared with the control (0 mg/L) and done in three replicates. The chosen concentrations represent 0.025 and 0.1 of the values of the calculated $EC_{50}$ (half maximal effective concentration) (Table 1). $EC_{50}$ was calculated by using Origin Pro® 2024b software based on the inhibition ratio after the preliminary experiment, where the two microalgal species exposed to a series of concentrations (control, 0.005, 0.01, 0.1, 1, 5, 10, and 20 mg/L) of TFL and NFL examined for 96 h in a static system according to U. S. Environmental Protection Agency protocol (EPA, 2002).

**Determination of total carbohydrate and protein contents**

Anthrone reagent method was utilized to determine the total carbohydrate (Badour, 1959). One mg of dry algal biomass was mixed with 1.25 mL of double-distilled water. For each sample, both blank and standard, 4 mL of anthrone reagent was inserted. They were then simmered in a boiling water bath for eight minutes and allotted to cool at room temperature for 10 minutes at 620 nm. The absorbance was measured in comparison to the blank. The amount of carbohydrates was stated as milligrams per gram of dry weight. The standard utilized was sucrose.

The Folin reagent was used to calculate the total protein content in accordance with the Lowry *et al*. (1951) method. One mg of fresh weight algal biomass was combined with one mL of 1N NaOH and boiled for ten minutes in a boiling water bath. Each milliliter of the extract was combined with five mL of reagent A, which was made by mixing one mL of freshly made 1%Na-K tartrate solution with 0.5% $CuSO_4$ into 50 mL of 2% $Na_2CO_3$ solution. The mixture was then allowed to sit at room temperature for ten min. After mixing the shaker with 5 mL of reagent B (Folin reagent), the mixture was once more incubated for 30 minutes at room temperature. At 650 nm, the absorbance was measured using the Folin reagent as a blank. The amount of protein was stated as milligrams per gram of fresh weight.

**Total lipid determination**

Sulfo-Phospho-Vanillian (SPV) method was conducted for total lipid determination (Anschau *et al*., 2017). By utilizing 6 mg of Vanillin (98%) dissolved in hot water (100 mL) and diluted with phosphoric acid (purity; 85%) to 500 mL, Phosphovanilin (PV) reagent was prepared. The prepared mixture was stored at 4 °C in a dark bottle until used. For lipid determination, an aliquot (0.41 mg) of fresh biomass from each microalga was suspended in distilled water (1 mL) and watered down, as required. Twenty microliters of the sample were mixed with 180 µL of concentrated sulfuric acid (purity; 98.0%) in test tube and incubated at 100 °C for 10 minutes. After cooling at room temperature, vanillin-phosphoric acid reagent (0.5 mL) was incorporated for color production. Then the mixture was cooled again after the incubation for 15 minutes at 37 °C. After 45 minutes of storage in the dark, the samples' absorbance at 530 nm was measured. The content was expressed as mg/g fresh weight.

**Fatty acids profile**
**Fatty acids extraction**

Fatty acids were determined by ultrasonic solid-liquid extraction (USE) technique (Kiluyla *et al*., 2015). Fifty mg of algal biomass were mixed with 20 mL of chloroform: methanol (1:1 v/v) and incubated at 60 °C for 30 minutes. Then, the samples were sonicated at 60 °C for 1 h. and filtered using PTFE filters; 0.25 µm pore size. The organic layer was evaporated to dryness, re-dissolved in 1 mL of acetone and 0.5 mL of 3.0 M methanolic HCl and heated at 60 °C for 1 h. The tubes were cooled at room temperature and partitioned with 1 mL of *n*-hexane three times. The combined extract was dried using anhydrous $Na_2SO_4$.

**Preparation of fatty acids methyl esters**

Fatty acid methyl esters were prepared from algal biomass according to the method of IUPAC, (2020) with slight modification. The vials were dried under stream of nitrogen, and the remaining films were dissolved in 2 mL of methanolic $H_2SO_4$ (2%). Then, the mixture was incubated at 90 °C for 90 minutes. After cooling, it was partitioned with petroleum ether (60-80 °C). The organic layer was decanted, filtered on 0.25 µm PTFE filter and employed for gas chromatographic-mass spectrometry (GC-MS) determination.

**Table 1.** Sublethal concentrations of traditional and nano-form of fluazifop-P-butyl based on $EC_{50}$ values.

| Microalgae | Fluazifop-P-butyl | $EC_{50}$ mg/L | 0.025 of $EC_{50}$ | 0.1 of $EC_{50}$ |
|---|---|---|---|---|
| *A. pyrenoidosa* | Traditional form | 10.91 | 0.273 mg/L | 1.09 mg/L |
| | Nano-form | 14.28 | 0.357 mg/L | 1.43 mg/L |
| *R. subcapitata* | Traditional form | 1.17 | 0.029 mg/L | 0.117 mg/L |
| | Nano-form | 4.76 | 0.119 mg/L | 0.476 mg/L |

### Gas Chromatography-mass spectrometry (GC-MS) analysis

Thermo Scientific Trace 1300 gas chromatography equipped with a Thermo Scientific TSQ 9000 Triple quadruple mass spectrometric, detector and capillary column TR FAM E (30 x 0.25 mm x 0.25 um) was used. The following circumstances were present when the samples were injected: With a split mode and split ratio of 1/100, helium was used as carrier gas at a flow rate of 1.0 mL/min. The injection volume was 1.0 µL, and the solvent delay was 1.57 min with a scanning range of 40 to 500 m/z and an ionizing energy of 50 eV, the mass spectrometric detector was run in electron impact ionization mode. The temperature of the ion source was 270 °C. Above auto-tune, the electron multiplier voltage (EM voltage) was kept at 1616 v. The temperature program started at 100 °C with 2 min hold then with rate 6.5 °C/min to 170 °C, followed by a rate of 2.8 °C/min to 200 °C with a 1 min hold. The injector temperature was set at 170 °C. The Wiley, NIST and Pest libraries were utilized for the identification of the separated peaks.

### Statistical analysis

Results are given as the mean of three replicates in each treatment. Using Origin Pro® 2024b software, $EC_{50}$ values were estimated. For these analyses, all the test series of FL on both the studied microalgae were combined to obtain sigmoidal fit of the concentration-response curve. The upper and lower parameters were fixed at 0 and 100 to indicate the percentage range for inhibition, and the center of the distribution is the estimated $EC_{50}$ value. To determine the effect of different concentrations within each treatment, the normality of the data distribution was first assessed using the Shapiro-Wilk test, which confirmed that the data were not normally distributed. Therefore, comparisons between concentrations were conducted using the non-parametric One-Way ANOVA (Kruskal-Wallis test). Subsequently, Dunn's post hoc test was applied to identify which groups of concentrations differed significantly. All findings were evaluated at the 5% significance level using Origin Pro® 2025b.

## RESULTS AND DISCUSSION

### Effect of TFL and NFL on total carbohydrate, protein and lipids of *Auxenochlorella pyrenoidosa* and *Raphidocelis subcapitata*

Results represent the total carbohydrate, protein and lipids in the treated cells of *A. pyrenoidosa* with 0.273 & 1.09 mg/L of TFL and 0.357 & 1.43 mg/L of NFL are shown in Figures 1, 2. Additionally, Results represent the total carbohydrate, protein and lipids in the treated cells of *R. subcapitata* with 0.029 & 0.117 mg/L of TFL and 0.119 & 0.437 mg/L of NFL are shown in Figures 3, 4.

Carbohydrate content in cells of *A. pyrenoidosa* treated with 0.273 & 1.09 mg/L of TFL increased by 51.87% and 69.19%, respectively in comparison to control. Also, carbohydrate contents in cells of *A. pyrenoidosa* treated with 0.357 & 1.43 mg/L of NFL increased by 47.14% and 73.47%, respectively compared to control. The maximum value of total carbohydrate (446.6 ± 22.3 mg/g dry weight) (95% CI:391.1-502.1) was logged in *A. pyrenoidosa* treated with 1.43 mg/L of NFL (Figure 2). In the case of *R. subcapitata,* carbohydrate content in cells treated with 0.029 & 0.117 mg/L of TFL increased by 52.09% and 78.87%, respectively compared to the control. Also, carbohydrate content in cells treated with 0.119 & 0.437 mg/L of NFL increased by 36.89% and 78.02%, respectively compared to control. Cells treated with 0.117 mg/L of TFL recorded the maximum value of total carbohydrate content (473.1± 3.38 mg/g dry weight) (95% CI:464.71-481.50) (Figure 3).

Total carbohydrate content increased significantly ($p < 0.05$) according to One-way Anova (Kruskal-Walli's test) in both the microalgae studied after exposure to TNF and NFL herbicide. This increase may be explained as an adaptive mechanism for the survival of microalgae under toxicant stress condition (Noaman *et al*., 2020). With this respect, our results are in accordance with Manikar *et al*. (2013) who showed a significant increase in the carbohydrate content of *Anabaena variabilis* after exposure to malathion herbicide. Also, glyphosate exposure increased total carbohydrates in *Chlorella sorokiniana*

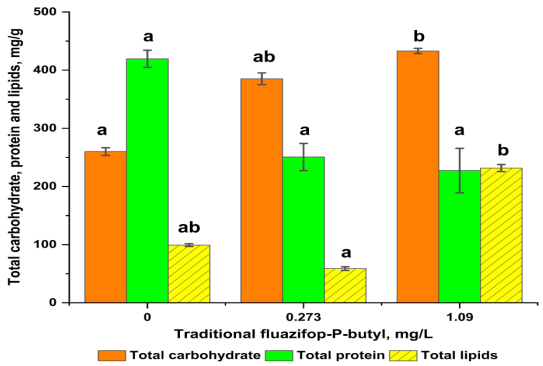


**Figure 1.** Effect of varied concentrations of traditional fluazifop-P-butyl on total carbohydrate (mg/g dry weight), total protein and total lipids (mg/g fresh weight) of *Auxenochlorella pyrenoidosa*. Error bars represent standard deviation (n=3). Different letters for the same-colored columns indicated a significant difference at p ≤ 0.05 according to Dunn's Test.

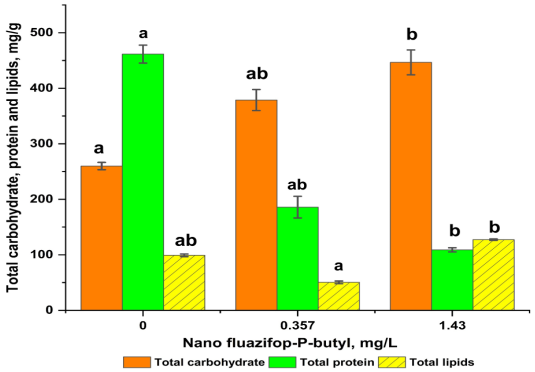


**Figure 2.** Effect of varied concentrations of nano fluazifop-P-butyl on total carbohydrate content (mg/g dry weight), total protein and total lipids (mg/g fresh weight) of *Auxenochlorella pyrenoidosa*. Error bars represent standard deviation (n=3). Different letters for the same-colored columns indicated a significant difference at p ≤ 0.05 according to Dunn's Test.

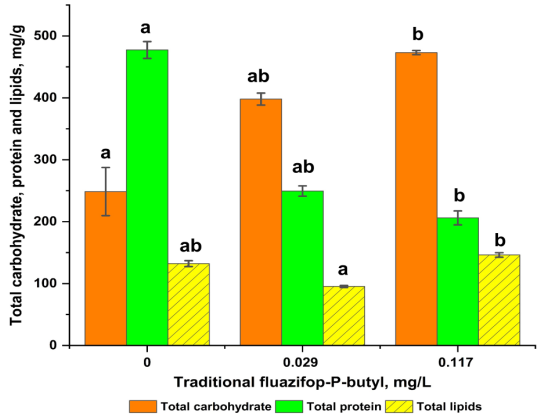


**Figure 3.** Effect of varied concentrations of traditional fluazifop-P-butyl on total carbohydrate content (mg/g dry weight), total protein and total lipids (mg/g fresh weight) of *Raphidocelis subcapitata*. Error bars represent standard deviation (n=3). Different letters for the same-colored columns indicated a significant difference at p ≤ 0.05 according to Dunn's Test.

by approximately 21% compared to the control (Jaiswal *et al*., 2020).

Protein content in cells of *A. pyrenoidosa* treated with 0.273 & 1.09 mg/L of TFL decreased by 40.56% and 44.70%, respectively compared to control. Also, protein content in cells of *A. pyrenoidosa* treated with 0.357 & 1.43 mg/L of NFL decreased by 60.84% and 76.38%, respectively compared to control. The lowest value of total protein (111.1 ± 3.70 mg/g) (95% CI: 101.92 - 120.30) was logged in cells that were treated with 1.43 mg/L of NFL (Figure 2). Concerning *R. subcapitata,* protein content in cells treated with 0.029 & 0.117 mg/L of TFL decreased by 47.15% and 57.41%, respectively compared to control. Also, protein content in cells treated with 0.119 & 0.437 mg/L of NFL decreased by 35.74% and 44.11%, respectively compared to control. The lowest value of total protein (239.32 ± 13.34 mg/g) (95% CI: 206.17-272.46) was logged in cells treated with 0.117 mg/L of TFL (Figure 3).

Protein content decreased in both the microalgae studied after exposure to herbicide in all treatments in comparison to the control. Data are statistically difference in the case of exposure of *R. subcapitata* to both NFL &TFL (p-value = 0.027) according to One-way Anova (Kruskal-Walli's test). Also, in the case of exposure of *A. pyrenoidosa* to NFL (p-value = 0.026) according to One-way Anova (Kruskal-Walli's test). On the other hand, data are not statistically difference in the case of exposure of *A. pyrenoidosa* to TFL (p-value > 0.05). There are numerous explanations for the decrease in protein synthesis. The first is the suppression of the synthesis of aromatic amino acids, which leads to the suppression of protein synthesis, nucleic acid metabolism, and the quantity of proteins linked to the photosystem II. The second is the use of aminotransferases to induce the breakdown of amino acids involved in the tricarboxylic acid cycle (Jaiswal *et al*., 2020). Lastly, changes in gene expression may be the cause of the decreased microalgal protein synthesis under herbicide-induced stress (Vivancos *et al*., 2011). The maximum value of total lipid (234.67 ± 6.08 mg/g) (95% CI: 206.17 - 272.46) was logged in cells of *A. pyrenoidosa* that were treated with 1.09 mg/L of TFL (Figure. 1). Concerning *R. subcapitata,* the highest value of total lipid (147.70 ± 3.66 mg/g) (95% CI: 138.62 - 156.78) was chronicled in cells treated with 0.117 mg/L of TFL (Figure 3). Total lipids increased by 136.22% in cells of *A. pyrenoidosa* treated with 1.09 mg/L of TFL compared to control. Also, lipids increased by 9.78% in cells of *R. subcapitata* treated with 0.117 mg/L of TFL compared

with control. This trend of rise could be attributed to their critical involvement in physiological stress tolerance in a range of organisms (El-Din, 2017). In addition, the herbicide quizalofop-p-ethyl (the same group of fluazifop-P-butyl) inhibits the activity of acetyl-CoA carboxylase (ACCasa) cytosolic increasing the substrate malonyl-CoA (precursor of the synthesis of fatty acids), causing accumulation of triacylglycerols (TAGs) (Chatuverdi *et al*., 2004; Bellou and Aggelis, 2012). Our findings are consistent with those of Yeh and Chang, (2011) and Sun *et al*. (2014) who found that nitrogen deprivation increased the lipid content of different algae. Furthermore, Malbezin *et al*. (2025) found that S-metolachlor boosted the lipids in the diatom *Gomphonema parvulum*. Data are statistically significant (p-value = 0.027) according to One-way Anova (Kruskal-Walli's test) in both the studied microalgae after exposure to TFL and NFL herbicide.

It is noteworthy to mention that *R. subcapitata* was more sensitive to TFL than NFL. High concentration (0.117 mg/L) of TFL increased carbohydrate and lipid contents by 0.5% and 15%, respectively, when compared to the high concentration (0.476 mg/L) of NFL. On the other hand, it has decreased protein content by 23.8%. Our findings are in accordance with Noaman *et al*. (2020) who found that traditional form of pendimethalin increased carbohydrate, lipids and decreased protein content than the nanoform of this herbicide. Also, Wang et al. (2022) stated that nanopesticides showed significantly lower toxicity on non-target species by more than 40% owing to controlled release of active ingredient when compared to traditional formulations.

### Effect of TFL and NFL on fatty acids composition of *Auxenochlorella pyrenoidosa* and *Raphidocelis subcapitata*

Table 2 shows the effect of different concentrations of TFL and NFL on the content of fatty acids fraction in *A. pyrenoidosa*. The total number of the recorded fatty acid fractions were 13 (8 saturated and 5 unsaturated). The total percentages of identified saturated fatty acids were 8.495, 6.334, 4.399 and 3.334 and of the unsaturated fatty acids were 18.990, 5.580, 3.657 and 4.966 in 0.273 mg/L, 1.09 mg/L of TFL, 0.357 mg/L and 1.43 mg/L of NFL, respectively. Meanwhile, the control percentages were 4.242 and 6.620 in saturated and unsaturated fatty acids, respectively. The saturated-to-unsaturated fatty acid ratio was 0.447, 1.135, 1.203, and 0.671 as compared to the control (0.641).

A heat map based on the content of the thirteen FAs in all the treatments was constructed (Figure 5). The control, traditional and nano-form of the herbicide were clustered into three groups, (control & TFL (1.09 mg/L)), (NFL (0.357 mg/L) & NFP (1.43 mg/L)) and (TFL (0.273 mg/L)). The traditional form of FL (TFL (0.273 mg/L)) was in a separate group due to the high content of palmitoleic acid, eicosenoic acid, linolenic acid and octadecanoic acid. The effect of different concentrations from TFL and NFL on the content of fatty acids fraction in *R. subcapitata* is given in Table 3.

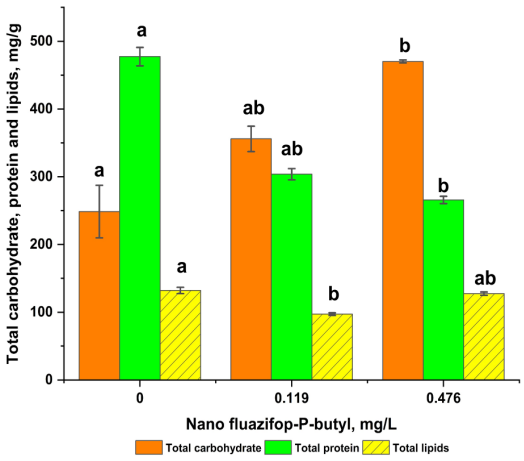


**Figure 4.** Effect of varied concentrations of nano fluazifop-P-butyl on total carbohydrate content (mg/g dry weight), total protein and total lipids (mg/g fresh weight) of *Raphidocelis subcapitata*. Error bars represent standard deviation (n=3). Different letters for the same-colored columns indicated a significant difference at $p \leq 0.05$ according to Dunn's Test.

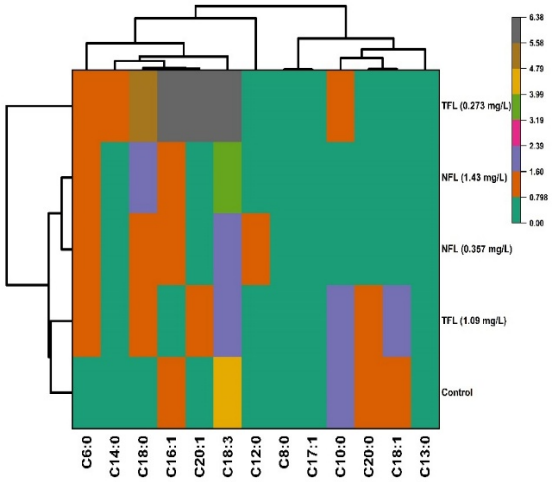


**Figure 5.** Heat map of fatty acids composition in *Auxenochlorella pyrenoidosa* treated with different concentrations of traditional fluazifop-*P*-butyl (TFL) and nano-form fluazifop-*P*-butyl (NFL).

**Table 2.** Effect of traditional fluazifop-P-butyl (TFL) and nano-form fluazifop-P-butyl (NFL) on fatty acids content (mg/g biomass) of *Auxenochlorella pyrenoidosa*.

| Fatty acid | Fluazifop-P-butyl herbicide | | | | | |
|---|---|---|---|---|---|---|
| | Control | TFL (0.237 mg/L) | TFL (1.09 mg/L) | NFL (0.357 mg/L) | NFL (1.43 mg/L) | p-value |
| Caproic acid (C6:0) | 0.103$^{a}$ | 1.500$^{b}$ | 0.950$^{ab}$ | 1.440$^{ab}$ | 1.210$^{ab}$ | 0.011* |
| Caprylic acid (C8:0) | BDL | BDL | 0.570$^{a}$ | BDL | BDL | 0.007* |
| Decanoic acid (C10:0) | 2.260$^{a}$ | 1.440$^{a}$ | 1.920$^{a}$ | BDL | BDL | 0.009* |
| Dodecanoic acid (C12:0) | BDL | BDL | 0.098$^{a}$ | 0.850$^{a}$ | BDL | 0.008* |
| Tridecanoic acid (C13:0) | 0.215$^{a}$ | BDL | BDL | BDL | BDL | 0.007* |
| Myristic acid (C14:0) | 0.124$^{ab}$ | 0.870$^{a}$ | 0.116$^{ab}$ | 0.530$^{ab}$ | 0.070$^{b}$ | 0.011* |
| Octadecanoic acid (C18:0) | 0.280$^{a}$ | 5.360$^{a}$ | 1.390$^{ab}$ | 1.410$^{ab}$ | 1.950$^{b}$ | 0.015* |
| Arachidonic acid (C20:0) | 1.260$^{a}$ | 0.108$^{a}$ | 1.290$^{a}$ | 0.109$^{a}$ | 0.104$^{a}$ | 0.035* |
| Total saturated | 4.242 | 9.278 | 6.334 | 4.399 | 3.334 | |
| Palmitoleic acid (C16:1) | 0.960$^{ab}$ | 6.490$^{a}$ | 0.390$^{b}$ | 0.850$^{ab}$ | 1.400$^{ab}$ | 0.009* |
| Heptadecanoic acid (C17:1) | BDL | BDL | 0.710$^{a}$ | 0.106$^{a}$ | BDL | 0.008* |
| Oleic acid (C18:1) | 1.610$^{a}$ | BDL | 1.980$^{a}$ | 0.151$^{a}$ | 0.416$^{a}$ | 0.008* |
| Eicosenoic acid (C20:1) | BDL | 6.430$^{a}$ | 0.890$^{a}$ | 0.350$^{a}$ | BDL | 0.008* |
| Linolenic acid (C18:3) | 4.050$^{ab}$ | 6.070$^{a}$ | 1.610$^{b}$ | 2.200$^{ab}$ | 3.150$^{ab}$ | 0.009* |
| Total unsaturated | 6.620 | 18.990 | 5.580 | 3.657 | 4.966 | |
| SFAs/USFAs ratio | 0.641 | 0.447 | 1.135 | 1.203 | 0.671 | |

BDL: Below detection limit. The mean of the three replicates was used to record all data. SFAs/USFAs ratio: proportion of saturated to unsaturated fatty acids. *Significant at p ≤ 0.05 according to One-Way ANOVA (Kruskal-Wallis test). According to the Dunns test, rows that share separate letters are significantly different at P ≤ 0.05.

**Table 3.** Effect of traditional fluazifop-P-butyl (TFL) and nano-form fluazifop-P-butyl (NFL) herbicide on FAs content (mg/g biomass) in *Raphidocelis subcapitata*.

| Fatty acids | Fluazifop-P-butyl herbicide | | | | | |
|---|---|---|---|---|---|---|
| | Control | TFL (0.029 mg/L) | TFL (0.117 mg/L) | NFL (0.119 mg/L) | NFL (0.476 mg/L) | p-value |
| Caproic acid (C6:0) | 1.340$^{a}$ | 0.854$^{a}$ | BDL | BDL | BDL | 0.008* |
| Caprylic acid (C8:0) | 0.143$^{a}$ | 0.128$^{a}$ | BDL | 0.105$^{a}$ | 0.116$^{a}$ | 0.009* |
| Dodecanoic acid (C12:0) | 0.750$^{a}$ | 0.526$^{a}$ | 0.701$^{a}$ | 0.405$^{a}$ | 0.424$^{a}$ | 0.015* |
| Tridecanoic acid (C13:0) | 0.215$^{a}$ | BDL | 0.159$^{a}$ | 0.113$^{a}$ | BDL | 0.008* |
| Myristic acid (C14:0) | 0.124$^{a}$ | 0.105$^{a}$ | 0.11$^{a}$ | 0.115$^{a}$ | 0.121$^{a}$ | 0.257 |
| Pentadecanoic acid C15:0 | 0.132$^{a}$ | BDL | BDL | BDL | 0.126$^{a}$ | 0.011* |
| Octadecanoic acid C18:0 | 0.237$^{a}$ | 1.310$^{a}$ | BD | 0.225$^{a}$ | 0.209$^{a}$ | 0.013* |
| Arachidonic acid (C20:0) | 0.110$^{a}$ | 0.094$^{a}$ | 0.099$^{a}$ | 0.088$^{a}$ | 0.089$^{a}$ | 0.076 |
| Total saturated | 3.051 | 3.017 | 1.076 | 1.051 | 1.085 | |
| Myristoleic acid C14:1 | 0.246$^{a}$ | 0.223$^{a}$ | BDL | 0.113$^{a}$ | BDL | 0.010* |
| Palmitoleic acidC16:1 | 0.374$^{ab}$ | 0.352$^{ab}$ | 0.325$^{a}$ | 0.369$^{ab}$ | 0.950$^{b}$ | 0.038* |
| Heptadecanoic acid (C17:1) | 0.152$^{a}$ | 0.840$^{a}$ | BDL | BDL | BDL | 0.008* |
| Oleic acid (C18:1) | 0.144$^{a}$ | 0.330$^{a}$ | BDL | BDL | 0.119$^{a}$ | 0.008* |
| Eicosenoic acid (C20:1) | 0.225$^{a}$ | 0.210$^{a}$ | BDL | BDL | BDL | 0.009* |
| Total unsaturated | 1.141 | 1.955 | 0.325 | 0.482 | 1.071 | |
| SFAs/USFAs ratio | 2.674 | 1.543 | 3.311 | 0.884 | 0.462 | |

BDL: Below detection limit. The mean of the three replicates was used to record all data. SFAs/USFAs ratio: proportion of saturated to unsaturated fatty acids. *Significant at p ≤ 0.05 according to One-Way ANOVA (Kruskal-Wallis test). According to the Dunns test, rows that share separate letters are significantly different at P ≤ 0.05.

The total number of the recorded fatty acid fractions were 13 fatty acids (8 fatty acids for saturated, 5 fatty acids for unsaturated). The total proportion of recognized saturated fatty acids was 3.017, 1.076, 1.051 and 1.085 and of the unsaturated fatty acids was 1.955, 0.325, 1.189 and 2.347 in 0.029 mg/L, 0.117 mg/L of TFL, 0.119 mg/L and 0.476 mg/L of NFL, respectively as compared to that of the control 3.051 and 1.141in saturated and unsaturated fatty acids, respectively. The ratio of saturated to unsaturated fatty acids was 1.543, 3.311, 0.884 and 0.462 as compared to the control of 2.674. For the individual fatty acids, all values of saturated fatty acids under both TFL and NFL treatments were shown lower than in control. A heat map based on the content of the thirteen FAs in all the treatments was constructed

(Figure 6). The control, traditional and nano-form of the herbicide were clustered into two groups, the first group (control & TFL (0.029 mg/L)) with high content of caproic acid and octadecanoic acid. The second group (TFL (0.117 mg/L)), NFL (0.119 mg/L) & NFL (0.476 mg/L)) contain the most undetected saturated and unsaturated fatty acids. Fatty acids are a prominent component of microalgal lipids, and their quantity and composition are affected by nutrition (nitrogen, carbon, phosphorus, iron, and trace metals) as well as environmental conditions (temperature, light and pH) (Juneja *et al*., 2013). Variations in total fatty acid content or the percentage of a certain fatty acid class may indicate that a pesticide is interfering with an organism's natural metabolism (Gonçalves *et al*., 2021). The increase of both monounsaturated and saturated fatty acids in the case of cells of *A. pyrenoidosa* subsequently being exposed to 0.273 mg/L of TFL and cells of *R. subcapitata* after exposure of 0.029 mg/L of TFL herbicide (e.g., C18:0 & C18:1). This increase could be interpreted as one of the self-defense mechanisms created by the cell against herbicide stress.

According to Olofsson *et al*. (2012) microalgae start accumulating C18 fatty acids, which act as electron sinks by using NAD(P)H and $O_2$ which decreases the cell stress. Triacylglycerols are commonly composed of high ratios of both saturated and monounsaturated fatty acids, whereas structural lipids (such as polar lipids) generally contain more polyunsaturated fatty acids. Our findings are consistent with the findings of Kumar *et al*. (2018) who found that *Chlorella sorokiniana* was able to synthesize triacylglycerol (TAGs) in its cellular compartments to mitigate the impacts of reactive oxygen species induced under malathion herbicide stress.

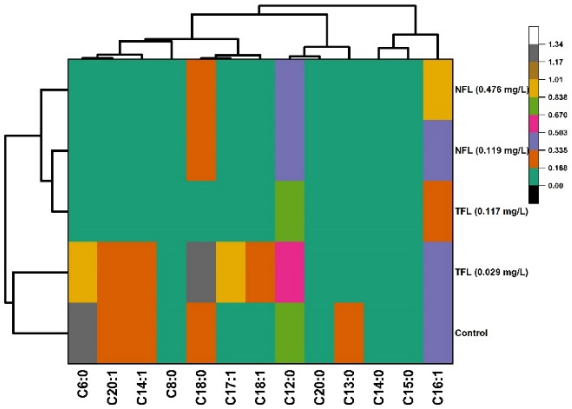


**Figure 6.** Heatmap of fatty acids composition of *Raphidocelis subcapitata* treated with different concentrations of traditional fluazifop-P-butyl (TFL) and nano-form fluazifop-P-butyl (NFL).

Unsaturated fatty acids in cells of *A. pyrenoidosa* decreased significantly due to treatment with 1.09 mg/L of TFL, 0.357 mg/L and 1.43 mg/L of NFL herbicide. Also, these unsaturated fatty acids decreased in cells of *R. subcapitata* when treated with 0.117 mg/L of TFL, 0.119 mg/L and 0.476 mg/L of NFL herbicide. Oxidation caused by FL herbicide on unsaturated fatty acids by the superoxide radicals ($O^{2-}$) and hydrogen peroxide ($H_2O_2$) may be the cause of this decrease in unsaturation (Luo *et al*., 2016). While the generation of reactive oxygen species (ROS) is a normal metabolic byproduct of aerobic metabolism in plants, excessive production usually indicates stress brought on by anthropogenic or environmental change (Sharma *et al*., 2012). Peroxy radicals and malondialdehyde are two ROS-induced lipid products. The peroxy radical is also an oxidative agent, generating extra peroxy radicals and a malondialdehyde molecule. Until antioxidant pathways effectively scavenge it, or cells die, the process will continue (Carve *et al*., 2017).

## CONCLUSION

As a model cell, 2 unicellular freshwater microalgae were investigated to clarify the influence of the traditional and nano-forms of herbicide. The sensitivity of *R. subcapitata* toward TFL and NFL was higher than *A. pyrenoidosa*. However, the sensitivity of *R. subcapitata* to TFL was higher than NFL. Both TFL and NFL affected carbohydrate, protein, lipid, and fatty acids composition of the studied microalgae. They have a positive effect on total carbohydrate content in the two microalgae studied, where it increased more than control. In contrast, TFL and NFL have a negative effect on protein content where protein content is decreased compared to the control. These consequences may compromise the safety of the habitat surrounding these microalgae. Accordingly, herbicides may have a significant concern for both environmental and human health. These chemicals, designed to target unwanted plants and weeds, can have unintended consequences when they enter ecosystems. Where they may affect water quality and aquatic organisms. Understanding the impact of herbicides on aquatic environments is critical for ensuring their safe and sustainable use.

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