The International Maritime Transport and Logistics (MARLOG) - ISSN 2974-3141
http://dx.doi.org/10.21622/MARLOG.2023.12.1.036

# Nutritional And Toxicological Importance Of Nickel, Copper And Zinc Elements In Spirulina Platensis

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## 1. ABSTRACT:

The presence of heavy metal ions in water is hazardous to one's health and the environment. Algae are frequently exposed to heavy metal pollution as a result of industrial waste dumping into water environments. *Spirulina platensis*, a significant kind of algae utilized commercially (especially for fish feeding) as a good source of protein, amino acids, minerals, vital unsaturated fatty acids, and a number of vitamins, was selected for this study because of its high nutritional value. This study examined how *S. platensis* responded to five different concentrations of the three heavy metals (nickel, copper, and zinc) in terms of its growth, fatty acid IR spectra, content, and total soluble protein profile. The remaining four doses (two higher and two lower) for each element were selected to evaluate the findings of 5 different concentrations of the three heavy metals because the EC50 for those three was almost at 2.0 mg/l. Compared to zinc and nickel, copper demonstrated a greater growth inhibitory impact as determined by optical density. $Cu^{2+}$ was more noticeable than $Zn^{2+}$ and $Ni^{2+}$ when compared to control cells in the IR spectra, which showed the creation of new molecules and the lack of other compounds. Total fatty acids decreased under stress at all concentrations examined, while saturated fatty acids outnumbered unsaturated fatty acids. $Cu^{2+}$ stress resulted in a more marked destructive effect of the heavy metal ions the protein profile than $Zn^{2+}$ or $Ni^{2+}$ stress.

***Keywords:*** *Heavy metals; Environmental Pollution; Spirulina platensis; Fatty acids; Infra Red; Protein profile.*

## 1. INTRODUCTION

The industrial revolution contributed to heavy metal pollution. Both the ecology of the environment and the life quality are impacted by pollution. Heavy metals play a vital role in many sectors, and they release a lot of pollution into the environment. Egypt has five lakes in the Mediterranean (Burullus, Mariut, Edku, Manzala, and Bardawil). They play a crucial role in supplying a priceless natural resource for the growth of fish. For a very long time, heavy metal contamination in the aquatic environment has been recognised as posing a serious hazard to aquatic life, especially fish. These lakes have the following degrees of pollution: Manzalah in the first order followed by Edku , Burollus , Bardawil and Mariut (**Saad, 2003**).

Being the base of the aquatic food web and the principal producers, phytoplankton is a varied collection of microbes that is essential for preserving species diversity, (**Hanan *et al.*, 2015**). The potential of microalgae bioenergy systems in terms of resources, energy, biofuel generation, and highvalue products has all been carefully examined. *Spirulina platensis* stands out among economically significant microalgae for its high protein content, pigment, and fatty acid, making it appropriate for application in animal and human feeding, (**Nethravathy *et al.*, 2019**). The two species that are

utilised commercially the most frequently are *Spirulina platensis* and *Chlorella vulgaris*. According to current estimates, the annual dry matter output of *chlorella* and *spirulina* in the world is 6600 and 12,000 tons, respectively (**Garcia *et al.*, 2017**). For *S. platensis*, values for lipid content range from 9 to 17 % DW, depending on the culture conditions in addition to protein., **(Piorreck *et al.*, 1984)**. Due to its great nutritional content, *spirulina* has become popular as a promising and useful feed additive. Furthermore, its rich phytochemicals have considerable anti-inflammatory and antioxidant properties, **(Abdel-Latif *et al.*, 2022).** Production of microalgae aims for the highest levels of biomass production and quality while minimizing nutrient shortages and other unfavourable growing situations. A total nitrogen measurement overestimates the genuine protein level since protein includes nitrogen along with other nitrogenous constituents such as fatty acids, amines, and cell wall material, **(Muys *et al.*, 2019)**. It is well known that microalgae are good heavy metal bio-accumulators, **(Arunakumara and Xuecheng, 2008; Kaamoush *et al.*, (2022)**. While certain metals, like As, Hg, Cd, Pb, and Ni, are poisonous, Zn, Cu, and Cr are necessary for human nutrition but can be hazardous if ingested in excess. Other metals, including Hg, As, Pb, Cd,and Ni, are toxic. The toxicity of heavy metals to individuals and the environment is a global issue. An effective bio-accumulator of a variety of heavy metals is *spirulina*, **(Sanjib, 2020)**. Nowadays, *Spirulina platensis* is frequently used to combat malnutrition, especially in young infants. Due to its high levels of protein, vitamins, minerals, healthy fatty acids, and other restorative phytonutrients like different active plant colours, these blue-green cyanobacteria microalgae are cultivated in temperate seas all over the world and are regarded as a functional food. *Spirulina* was recognised by the World Association for Applied Microbiology as a possible food source in 1967, **(Lupatini *et al.*, 2017)**. *Spirulina platensis* has a high nutritional value because to its abundance of amino acids and considerable amounts of -linoleic acid and other unsaturated fatty acids. Calcium, potassium,iron, magnesium, manganese, phosphorus, selenium, and zinc are among the minerals that may be found in it. *Spirulina* contains a number of vitamins, including E, B1, B2, and B12, **(Pyne *et al.*, 2017).**

Over the last ten years, the nutritive benefits of *spirulina* in aquaculture feeds has been carefully examined as a fishmeal substitute or as a functional feed supplement to enhance fish growth and performance. Due to its high protein content (up to 70%), enormous amount of vital fatty acids, antioxidant pigments (phycobiliproteins and beta carotene), and polysaccharides, commercial *spirulina* production has grown in popularity for use in medical products, nutritional omega-3s for humans and animals, and cosmetics. *Spirulina* can replace animal-derived proteins in aquafeed for aquaculture, **(Ragaza *et al.*, 2020).** These fatty acids, which have significant structural, chemical, and functional functions in human metabolic pathways, can be produced by *spirulina*. Along with serving as a source of energy, fatty acids, especially omega-3 and omega-6, have emerged as a common reference point for conditions including cardiovascular, neurological, and endocrinologic ailments, (**Zheng *et al.*, 2012**).

## 2. MATERIALS AND METHODS

### *I-Growth measurement:*

*Spirulina platensis* was cultivated in **(Zarrouk, 1966)**. Growth of *Spirulina platensis* can be best determined by optical density (degree of turbidity) measurements. Using a Perkin Elmer (Lambada 1) ultra violet spectroscopy with a 560 nm wave length, this approach measures the percentage of light transmittance (T) that has changed in comparison to control tubes that have not been infected and are set at 100% T. The following equation was used to compute the optical density(**Robert, 1979**):

**Optical density ( O.D.) = log $I_0/I$**

Where: I= the transmittance of sample.

$I_{O=}$ the transmittance of blank adjusted to read 100%.

### *II-Preparation of different heavy metal concentrations*

$NiCl_2$, $CuSO_4.5H_2O$, and $ZnCl_2$ were purchased from Algmhoria Co. in Alexandria, Egypt. The three heavy metals nickel, copper, and zinc were chosen for this inquiry due to their ubiquity in industrial waste water and their negative impact on the ecosystem. Stock solutions for the chosen heavy metals were prepared by dissolving their salts in double-distilled water and sterilising the mixture using 0.2-m nitrocellulose membranes. By dilution with doubly distilled water, the various concentrations of selected heavy metals employed in the metal bioassays were created. The three heavy metals' EC50 was discovered to be around 2.0 mg/l. We selected four concentrations—two higher and two lower—for each of the three heavy metals in order to compare the outcomes of five distinct concentrations.

### *III- IR Measurements of cells tested*

The density of cell suspensions required to produce spectra with a good signal was determined in preliminary trials. According to **Kansiz *et al.*, (1999)** a known volume of algal culture pellets were treated with Lugol's iodine solution. The stage of the infrared microscope was set up with the dried cells so that spectral data could be collected for IR analysis. The spectra were collected using a ratio recording infrared spectrophotometer, the Perkin Elmer 1430. The absorbance spectra were collected between 4000 and 500 cm-1 and averaged using 10 scans.

## IV-Measurements of total lipid extraction

The examined alga Spirulina's total lipid content was extracted, as according **Bligh and Dyer (1959).** By adding 50 ml of a chloroform and methanol mixture (2: 1 v/v) to 50 ml of the algal cells in a separate funnel, the total lipids were completely extracted. After two layers formed, the aqueous layer containing the remaining cells was discarded, and the other layer containing the lipid extracts was washed with a 0.4% $MgCl_2$ solution (100 ml) before being repeatedly washed with distilled water (100 ml) For acidification, this extract was then combined with 1.5 N HCl. In order to separate fatty acids from oils, acidified extract was added to a separating funnel with 50 ml of 1n-hexan and 50 ml of newly made sodium carbonate. In order to re-acidify this mixture, 2 ml of 1.5 N HCl was added. A rotary evaporator was used to evaporate the filtrate after the hexane layer, which contained the fatty acids, was separated from the water layer and dried over anhydrous $Na_2SO_4$ to remove water droplets.

The resulting residue is a representation of all lipids. The vial was cooked in an oven at 90°C for 90 minutes before being submerged in a stream of nitrogen. The methyl ester of each vial was extracted three times using 5 ml of petroleum ether after the vial was cooled to room temperature and 10 ml of distilled water was added. Before detecting the fatty acid methyl esters, the three petroleum ether extracts were mixed and reduced in volume using a stream of nitrogen. On a gas chromatography device, all studies for the identification of fatty acids fractions were carried out.

## V-Measurements of protein profile

By boiling dialysis tubing (cut into small lengths of about 15 cm each) for 15 minutes in a solution of 1% sodium bicarbonate and 10-3 M Na2EDTA, protein profiles were calculated. After centrifuging 10 ml of culture, the freshly frozen cell pellet of algal material was obtained. It was then homogenised by grinding it in a mortar with quartz sand and a small amount of 0.5 M Tris-HCl buffer (PH 7.2). The solution was spun at 500 rpm for 10 minutes to concentrate the supernatant, which was then concentrated over a bed of sucrose in pre-activated dialysis tubing.

## RESULT AND DISCUSSION A- Growth:

The optical density (O.D.) of *Spirulina platensis* is an important growth parameter that is used as a risk indicator for various contaminants, particularly heavy metals. According to the results obtained after 8 days of culturing, Around 2.0 mg/L was the effective concentration (EC50) of nickel, copper, and zinc (different experiments have been done to determined effective concentration of the three examined heavy metals).

As a result, in this study five different concentrations were chosen (for each element besides control. Copper had a stronger inhibitory effect on growth than nickel and zinc at all concentrations tested. Algal growth was slowed down as copper concentration rose, while it was stimulated at the minimum concentration (1.0 mg/L). Our results are in agreement with **(Budi et al., 2020),** who found that the extraordinary growth rate of *Spirulina platensis* was reported by who noted that the treatment with the addition of 1 mg/L of heavy metal is necessary for boosting growth, but the greater the concentration of Cu provided, the lower the density of *Spirulina platensis*. We found that lower quantities of zinc and nickel enhanced development of Spirulina platensis and that they are regarded growth accelerators of *Spirulina platensis* when utilised at low concentrations. $Zn^{2+}$ ions were more hazardous than $Ni^{2+}$ ions. These results are consistent with those of **Meenakshi *et al.*, (2007)** who found that copper toxicity, which was greater than zinc, caused a considerable decline in growth in *Spirulina platensis* culture.

Heavy metal toxicity may be indicated by alterations in the growth environment and a decline in the development of the test microorganism. At low concentrations of zinc and copper, **Akbarnezhad *et al.*, (2019)m**'s research revealed that the optical density of *Spirulina platensis* culture gradually increased. *Spirulina platensis*, on the other hand, has a strong capacity in biosorption and bioaccumulation for heavy metal ions, making it a desirable option for environmental bioremediation, according to **Zinicovscaia *et al.*, (2021)**. They observed that it has a higher bioaccumulation capacity for Zn than Ni and Cu ions.

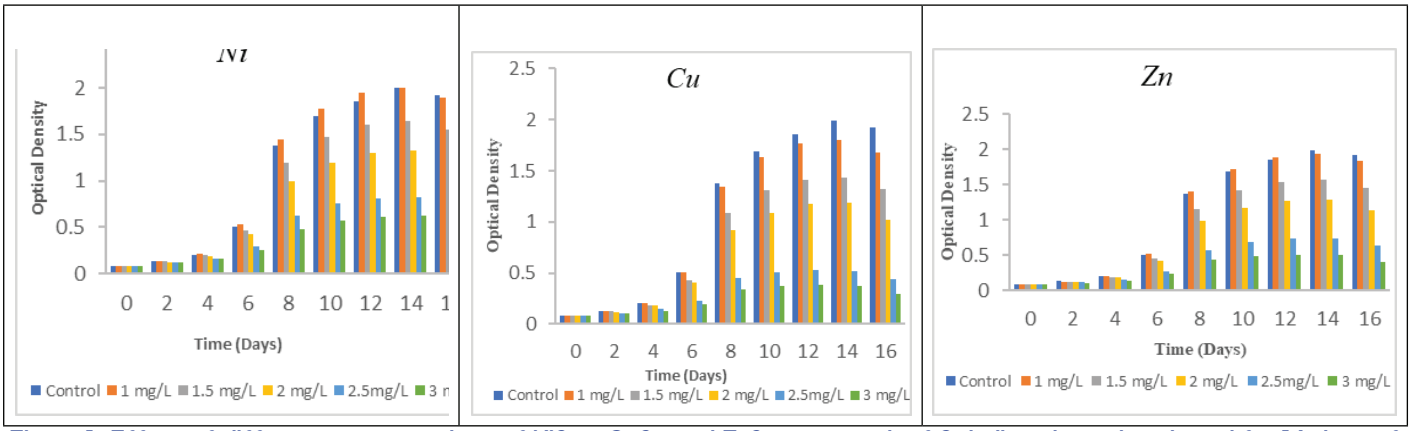


*Figure 1: Effect of different concentrations of Ni2+, Cu2+ and Zn2+ on growth of Spirulina platensis cultured for 16 days of culturing measured in optical density.*

http://dx.doi.org/10.21622/MARLOG.2023.12.1.036 Vol. 12, Iss. 1, August 2023

## B- IR Spectra:

Infrared spectroscopy is quick and reliable, needs a small sample size, and has a straightforward sample preparation process, which are all benefits over other conventional methods of biochemical analysis **(Kansiz *et al*., 1999).** Based on research on *S. platensis* entire cells, compartments, and biomolecules in the 4000-250 Cm-1 range, infrared spectra of the complete cell contents showed band assignments. The data show that there are several spectral regions that potentially explain the chemical variations in this species (**Fig.2, 3 and 4**).

When *S. platensis* was cultivated for 8 days under the stress of various concentrations of $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ ion metals, the obtained infrared peaks of the primary cell constituents were examined. When compared to the control, it is evident that some peaks disappeared, others appeared new, and still others remained the same. The new peaks that developed when these three components were stressed could have resulted from side chain position alterations or the breakdown of some compounds with high molecular weights into those with lower molecular weights. These ideas are consistent with those obtained by **Al-Osaimi, (2010)**. Infrared spectroscopy is quick and reliable, needs a small sample size, and has a straightforward sample preparation process, which are all benefits over other conventional methods of bioassay **(Kansiz *et al.*, 1999).** Based on research on S. platensis entire cells, compartments, and macromolecules in the 4000-250 Cm-1 range, infrared spectra of the complete cell contents showed band assignments.

The asymmetric and symmetric C-H of the methylene groups were present in the disappearing peaks, which disappeared at frequency 4500-4000 and 3500-3000 cm-1, respectively. The number of peaks is the same for Cu concentrations of 1.0 and 1.5 mg/l (14 peaks), but it is lessened at Cu concentrations of 2.0, 2.5, and 3.0 mg/l (13, 12 and 7 peaks, respectively). The number of gone peaks was 5, 5, 6, 8 and 7 peaks, with respective concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l. At doses of 1.0 to 2.0 mg/l, there were five new peaks that emerged, and at 2.5 mg/l, there were six new peaks. Cu concentration 3.0 mg/l produced just one peak.

Accordingly, as compared to untreated cells, new peaks in the spectra of the studied algae species S. platensis cultured at various dosages of Ni, Cu, and Zn emerged while other components vanished. The disappearance of some compounds, the protracted process of their synthesis, the movement of some side chains within a single molecule, and/or the dissociation of complex compounds into simpler ones may be the causes of these peaks. Our findings are consistent with those made by **El-Agawany and Kaamoush (2022),** they noted that the important cell components of *Dunaliella tertiolecta* procured infrared peaks demonstrated the development of new peaks and the damage of others, revealing changes in cell components brought on by the presence of different concentrations of zinc element.

The new peaks, which correspond to the amides associated with proteins as well as the function groups of other amides from lipids and fatty acids, emerged in all of the examined elements at frequencies between 2500 and 1000 cm-1 (**Williams and Feleming 1996**). When $Ni^{2+}$, $Zn^{2+}$, and $Cu^{2+}$ were added to control and modified cultures, more peaks at frequencies between 1500 and 1000 cm-1 occurred than in any other concentration examined. These frequencies correspond to the phosphodiester backbone of nucleic acids and the amides associated with protein (**Noctor and Foyer, 1998**).

Last but not least, it can be concluded that $Cu^{2+}$ metal ions are more hazardous than $Ni^{2+}$ and $Zn^{2+}$ metal ions, and the degree of stress is mostly determined by the element's type, concentration, and duration of culture. This has been proven by the findings of (**El-Sheikh *et al.*, 1999**), which showed that the type of element and its concentration affect the deleterious effects of heavy metals.

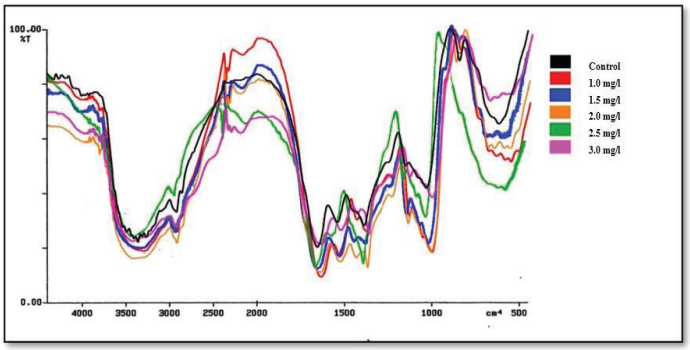


*Figure 2: Infrared spectra of Spirulina platensis cell components grown for 8 days with various Ni2+ concentrations.*

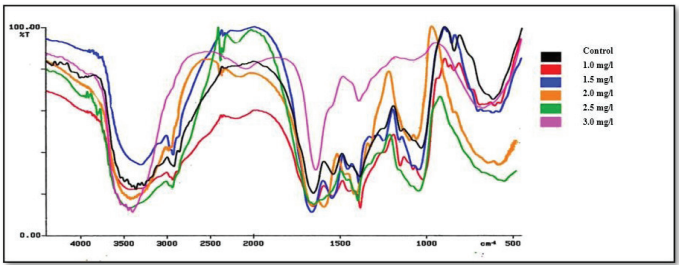


*Figure 3: Infrared spectra of Spirulina platensis cell components grown for 8 days with various Cu2+ concentrations.*

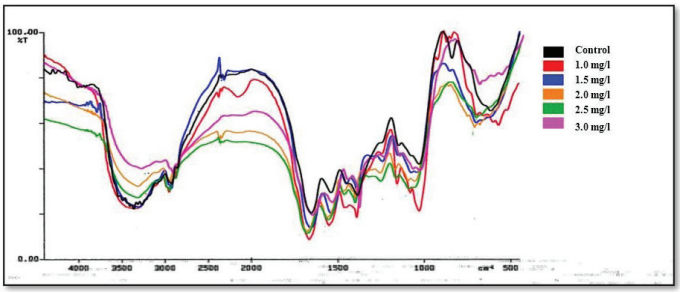


*Figure 4: Infrared spectra of Spirulina platensis cell components grown for 8 days with various Zn2+ concentrations.*

## C- Fatty acids:

The development and survival of aquaculture are correlated with the fatty acid composition of dietary microalgae. Lipid synthesis typically varies between genera, species, and strains of microalgae, which is a well-known fact. But in healthy phytoplankton, total lipid fractions range widely, from less than 1% to more than 40% of dry weight, (**Dubinsky *et al.*, 1978**). *Spirulina* contains a high concentration of highly valuable phytocompounds with distinct functional properties, such as omega3&6, carotenoids, and phenolic acids (**Pyne *et al.*, 2017**). Certain disorders, like coronary heart disease, can be prevented and treated in part by fatty acids, (**William, 2000**). Both the overall quantity of lipids and the relative concentrations of fatty acids can be impacted by environmental factors. Additionally, the lipid category and fatty acid content of microalgal cells can vary greatly depending on the stage of development, (**El-Maghrabi 2002**).

Regarding the impact of various $Ni^{2+}$ ion concentrations (1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) on the content of the 3 groups of fatty acid fractions (saturated, mono-, and poly- unsaturated fatty acids) in *S. platensis*, it is evident that $Ni^{2+}$ ions have a weakly harmful effect on the content of fatty acids in S. platensis when compared to control. In comparison to control, the level of saturated fatty acids rose by 12.36, 10.01, 10.66, 9.02, and 18.40% at each of the tested Ni doses. Additionally, the saturated fatty acids (C20:0 and C21:0) entirely vanished at concentrations 2.0 and 2.5 mg/l $Ni^{2+}$. However, the effects of the various Ni ion concentrations led to an increase in total saturated fatty acids.

It is clearly obvious that all three groups of fatty acids are significantly impacted, especially at high concentrations of the elements, based on the total fatty acids content of the examined alga *S. platensis* after 10 days of incubation in relation to the five concentrations of the selected elements. However, depending on the category of fatty acids, these metals had different negative impacts. Therefore, the total content of the three types of fatty acids decreased when the element's concentration was increased. It was determined that this drop was quite substantial. It was discovered that the harmful effects of $Ni^{2+}$, $Zn^{2+}$, and $Cu^{2+}$ were concentration-dependent, meaning that the toxic effect increased as the element concentration increased. **El-Sheikh *et al.* (1999)** recorded that toxicity of the element was a concentration dependent. $Cu^{2+}$ concentration had a higher negative impact on *Spirulina platensis* than $Zn^{2+}$ and $Ni^{2+}$ concentrations. The toxic effect of all fatty acid groups ranged from highly significant at low concentrations to extremely significant at high concentrations. The three tested heavy metals inhibited the synthesis of all groups of fatty acids at higher concentrations, but the degree of inhibition was more pronounced at higher concentrations, particularly in the content of polyunsaturated fatty acids. The results indicated that the toxic effect of the tested element on fatty acid content in *S. platensis* was more pronounced in the case of mono- and poly-unsaturated fatty acids than saturated fatty acids.

At the tenth day of culture, the organism normally synthesizes 27 fractions of fatty acids. **Dempester and Sommerfeld, (1998)** observed that nutrient deficits may result in an increase in cell lipid content, and as a result, older, healthier cultures of cells have fatty acid content that is higher than cells grown during the lag phase of growth. The two most dominant ones were the mono-unsaturated fatty acid C18:1 and the poly-unsaturated fatty acid C18:2. **Cohen,(1991)** reported that polyunsaturated fatty acids including lindonic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid are all abundant in microalgae. Numerous authors have indicated that linolinic acid (C18:2) fatty acid is necessary for the survival and development of a variety of young aquaculture organisms, (**El-Maghrabi, 2002**).

The overall level of saturated fatty acids decreased by 8.51% compared to control at 1.0 mg/l $Cu^{2+}$ concentration. These findings could support previous findings about growth, when an organism showed limited growth at the same concentration compared to the control. At concentration (1.0 mg/l) of $Cu^{2+}$, the saturated fatty acid C6:0 jumped by 287.56% compared to the control. The findings demonstrated that mono-unsaturated fatty acids dropped at all concentrations examined, though at varying rates depending on the element's concentration. Despite the fact that total poly-unsaturated fatty acids increased in response to all of the Cu concentrations studied, C20:5 was not detected at concentrations 2.5 and 3.0 mg/l, C20:3 at control and at concentration 3.0 mg/l, C20:2 and C22:6 were not detected at concentrations 1.5, 2.5, and 3.0 mg/l. C22:2 was detected at concentrations of 1.0 and 2.0 mg/l but not at control, 1.5, 2.5, or 3.0 mg/l. However, the total fatty acid concentration decreased at all concentrations tested, (**Fig. 5- 10**).

It is evident when considering the five tested concentrations of $Zn^{2+}$ on the content of the 3 groups of fatty acids (saturated, mono-, and poly-unsaturated fatty acids) in S. platensis that the total of the 3 groups of fatty acids increased at concentrations of 1.0, and 1.5 mg/l $Zn^{2+}$ (4.993 and 0.006% over control), whereas at concentrations 2.0, 2.5, and 3.0 mg/l Zn the total content decreased by 0.009, 0.009 and 0.005% below control, respectively. Under all of the Zn ion concentrations that were evaluated, there was a rise in the quantity of saturated fatty acids, and the percentage of the increase varied depending on the $Zn^{2+}$ concentration. In regard to mono-unsaturated fatty acids, our findings are consistent with those of **Alam *et al.*, (2010)** and **Balaji, (2015)** who observed that By interfering with chloroplast structure and result in changes in fatty acid content, heavy metals have the ability to influence the rate of photosynthesis. In addition, (**El-Agawany and Kaamoush, 2022**) found a discovery that confirms our findings they found

that overall concentration of the three fatty acid groups was decreased when zinc was increased in *Dunaliella tertiolecta* cultures, and the harmful effects of $Zn^{+2}$ were more evident for mono- and poly-5-unsaturated fatty acids than for saturated ones. **Dempester and Sommerfeld (1998)** noted that increasing the $MgCl_2$ content in the culture medium had a discernible impact on some diatoms' ability to produce neutral lipids. The presence of divalent metal cations, particularly magnesium ($Mg^{2+}$), was shown to be necessary for the activity of acetylCoA carboxylase, an enzyme used early in the synthesis of fatty acids, according to **Roessler (1989),** The same author noted decreased acetyl-CoA activity in the presence of only manganese ($Mn^{2+}$) and no acetyl-CoA activity in the presence of only cobalt ($Co^{2+}$). Increased lipid yield was observed with increasing salt concentration, which may cause physiological stress in *Botryococcus braunii* and *Isochrysis* species (**Ben-Amotz *et al*. 1985**) and in *Chlorella* species (**Tadros 1985**). **El-Maghrabi, (2002).** Nutrient limitation was identified as one of the major factors that enhanced lipid biosynthesis. The same results were achieved in our study. Cyanobacteria, on the other hand, do not exhibit significant changes in their lipid content and fatty acid composition in response to nitrogen supply (**Becker, 2004**). Nitrogen limitation was discovered to be an effective method of increasing lipid content, primarily at the expense of protein, (**Piorreck *et al.*, 1984**).

**Simonopoulos, (1991)** revealed that microlagae were a good source of Omega-3 fatty acids, which are protective against chronic illnesses like cancer, diabetes, and coronary heart disease. **Chu and Dupuy, (1980)** concluded that effects on fatty acid desaturation pathways may be responsible for variations in the relative quantities of polyunsaturated fatty acids. **Xu *et al*. (1997 and 1998),** reported that a decrease in membrane fluidity and permeability may be the cause of the decline in polyunsaturated fatty acid fractions. According to **Dowidar (1983),** in stressful circumstances, saturated fatty acids predominated over unsaturated ones. Our findings also indicated the same conclusion.

*Table 1: The effect of varying Ni2+ levels on the fatty acid fraction content (µg/ml) of Spirulina platensis grown for 10 days.*

| Fatty acids | | Control | Different $Ni^{2+}$ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | |
| | C6:0 | 0.789 | 1.488 | 1.112 | 8.644 | 9.644 | 12.312 |
| | C8:0 | 1.360 | 2.477 | 0.755 | 0.435 | 0.263 | 0.695 |
| | C10:0 | 0.416 | 1.056 | 0.423 | 0.241 | 0.192 | 0.430 |
| | C11:0 | 0.915 | 1.486 | 0.890 | 0.583 | 0.469 | 0.539 |
| | C12:0 | 0.336 | 0.924 | 0.564 | 0.256 | 0.273 | 0.403 |
| | C13:0 | 2.640 | 3.644 | 2.592 | 2.018 | 1.384 | 1.596 |
| | C14:0 | 2.020 | 2.032 | 0.320 | 1.257 | 1.480 | 1.661 |
| | C15:0 | 1.232 | 1.158 | 1.067 | 0.882 | 0.606 | 0.669 |
| | C16:0 | 35.324 | 40.647 | 47.297 | 43.171 | 40.929 | 41.181 |
| | C17:0 | 0.161 | 0.046 | 0.333 | 0.311 | 0.308 | 0.269 |
| | C18:0 | 9.428 | 4.333 | 5.113 | 3.912 | 5.243 | 5.345 |
| | C20:0 | 0.484 | 0.148 | 0.457 | -- | -- | 0.547 |
| | C21:0 | 0.658 | 0.216 | 0.421 | -- | -- | 0.375 |
| Total | | 55.763 | 59.657 | 61.346 | 61.71 | 60.791 | 66.022 |
| % of increase | | --- | (+)6.98 | (+)10.01 | (+)10.66 | (+)9.02 | (+)18.40 |
| | C14:1 | 6.571 | 14.182 | 1.626 | 9.475 | 10.214 | 10.163 |
| | C15:1 | 2.115 | 1.786 | 2.115 | 1.831 | 1.601 | 2.578 |
| | C16:1 | 2.022 | 3.652 | 4.464 | 4.642 | 4.672 | 2.793 |
| | C17:1 | 0.394 | 0.131 | 0.354 | 0.222 | 0.216 | 2.449 |
| | C18:1 | 16.063 | 2.868 | 6.830 | 4.267 | 7.046 | 5.041 |
| | C20:1 | 0.253 | 0.435 | 0.397 | -- | -- | -- |
| | C22:1 | 2.372 | 4.072 | 1.937 | 0.487 | 0.400 | 1.422 |
| Total | | 29.79 | 27.128 | 17.723 | 20.924 | 24.149 | 24.446 |
| % of decrease | | --- | (-)8.94 | (-)40.51 | (-)29.76 | (-)18.94 | (-)17.94 |
| | C18:3 | 5.941 | 4.090 | 9.610 | 8.723 | 6.704 | 4.305 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| - | C18:2 | 7.785 | 4.870 | 8.327 | 7.276 | 6.307 | 4.155 |
| | C20:5 | 0.155 | 0.152 | 0.368 | 0.539 | 0.271 | 0.166 |
| | C20:3 | -- | 0.415 | 0.766 | 0.500 | 0.781 | 0.175 |
| | C20:2 | 0.275 | 0.824 | 0.726 | -- | 0.483 | -- |
| | C22:6 | 0.292 | 1.147 | 0.371 | 0.330 | 0.260 | 0.131 |
| | C22:2 | -- | 1.251 | 0.763 | -- | 0.255 | 0.600 |
| Total | | 14.448 | 12.749 | 20.931 | 17.369 | 15.061 | 9.53 |
| % of increase or decrease | | --- | (-)11.76 | (+)44.87 | (+)20.22 | (+)4.24 | (-)34.04 |
| Grand Total content | | 100.001 | 99.533 | 100.000 | 100.003 | 100.001 | 100.091 |
| % of increase or decrease | | --- | (-)0.47 | (-)0.009 | (+)0.002 | 0.000 | (+)0.090 |

*Table 2: The effect of varying Cu2+ levels on the fatty acid fraction content (µg/ml) of Spirulina platensis grown for 10 days.*

| Fatty acids | | Control | Different $Cu^{2+}$ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | |
| | C6:0 | 0.787 | 3.053 | 4.631 | 0.511 | 1.050 | 1.510 |
| | C8:0 | 1.361 | 1.061 | 1.079 | 1.849 | 0.276 | 1.877 |
| | C10:0 | 0.416 | 0.362 | 0.641 | 0.474 | 0.165 | 0.446 |
| | C11:0 | 0.915 | 0.944 | 0.918 | 0.698 | 1.118 | 0.771 |
| | C12:0 | 0.336 | 0.373 | 0.781 | 0.641 | 0.541 | 0.593 |
| | C13:0 | 2.642 | 2.459 | 2.727 | 1.921 | 3.674 | 2.864 |
| | C14:0 | 2.018 | 1.737 | 2.050 | 1.661 | 2.161 | 1.396 |
| | C15:0 | 1.234 | 0.243 | 0.222 | 0.881 | 0.238 | 1.021 |
| | C16:0 | 35.324 | 31.800 | 38.408 | 40.202 | 39.111 | 39.029 |
| | C17:0 | 0.161 | 0.266 | 0.377 | 0.337 | 0.273 | 0.548 |
| | C18:0 | 9.427 | 6.878 | 7.392 | 7.108 | 6.786 | 5.173 |
| | C20:0 | 0.484 | 0.762 | -- | 0.791 | 0.296 | -- |
| | C21:0 | 0.658 | 1.077 | 0.580 | 0.797 | 0.758 | 0.663 |
| Total | | 55.763 | 51.015 | 59.808 | 57.87 | 56.447 | 55.89 |
| % of increase or decrease | | --- | (-)8.51 | (+)7.25 | (+)3.78 | (+)1.23 | (-)1.21 |
| | C14:1 | 6.573 | 2.871 | 8.377 | 6.496 | 8.017 | 7.763 |
| | C15:1 | 2.113 | 0.744 | 2.179 | 1.868 | 1.965 | 1.100 |
| | C16:1 | 2.020 | 4.377 | 3.500 | 3.332 | 4.901 | 4.028 |
| | C17:1 | 0.396 | 0.343 | 0.429 | 0.365 | 0. 459 | 2.489 |
| - | C18:1 | 16.063 | 12.787 | 9.629 | 10.060 | 6.107 | 10.710 |
| | C20:1 | 0.253 | 0.293 | -- | 0.297 | 0.439 | -- |
| | C22:1 | 2.372 | 3.244 | 1.181 | 3.353 | 1.665 | 1.519 |
| Total | | 29.79 | 24.659 | 25.295 | 25.771 | 23.547 | 27.609 |
| % of decrease | | --- | (-)17.22 | (-)15.09 | (-)13.49 | (-)20.96 | (-)7.32 |
| - | C18:3 | 5.941 | 7.735 | 6.646 | 6.461 | 8.682 | 7.887 |
| | C18:2 | 7.785 | 8.682 | 7.805 | 7.297 | 11.053 | 8.611 |
| | C20:5 | 0.157 | 0.244 | 0.255 | 0.207 | -- | -- |
| | C20:3 | -- | 0.756 | 0.190 | 0.277 | 0.272 | -- |
| | C20:2 | 0.272 | 0.823 | -- | 0.647 | -- | -- |
| | C22:6 | 0.293 | 0.496 | -- | 1.042 | -- | -- |
| | C22:2 | -- | 0.072 | -- | 0.234 | -- | -- |
| Total | | 14.448 | 18.808 | 14.896 | 16.164 | 20.007 | 16.497 |
| % of increase | | --- | (+)30.18 | (+)3.10 | (+)11.88 | (+)38.48 | (+)14.18 |
| Grand Total content | | 100.001 | 94.482 | 99.999 | 99.995 | 100.001 | 99.196 |
| % of decrease | | --- | (-)5.52 | (-)0.002 | (-)0.006 | 0.000 | (-)0.805 |

*Table 3: The effect of varying Zn2+ levels on the fatty acid fraction content (µg/ml) of Spirulina platensis grown for 10 days.*

| Fatty acids | | Control | Different Zn2+ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | |
| | C6:0 | 0.786 | 1.646 | 6.766 | 36.453 | 4.975 | 1.573 |
| | C8:0 | 1.362 | 1.465 | 1.173 | 3.058 | 1.605 | 0.960 |
| | C10:0 | 0.416 | 1.021 | 0.867 | 4.290 | 1.035 | 0.062 |
| | C11:0 | 0.915 | 1.422 | 0.734 | 1.948 | 0.925 | 1.014 |
| | C12:0 | 0.336 | 1.282 | 0.770 | 0.218 | 0.436 | 0.585 |
| | C13:0 | 2.642 | 4.808 | 1.618 | 5.241 | 3.402 | 2.992 |
| | C14:0 | 2.018 | 0.694 | 1.433 | 4.630 | 2.253 | 1.936 |
| | C15:0 | 1.234 | 2.074 | 0.746 | 2.082 | 1.638 | 2.132 |
| | C16:0 | 35.324 | 36.644 | 33.581 | 15.877 | 32.458 | 35.601 |
| | C17:0 | 0.161 | 5.494 | 0.137 | 0.800 | 0.3210 | 0.208 |
| | C18:0 | 9.427 | 6.325 | 7.629 | 3.153 | 9.935 | 8.406 |
| | C20:0 | 0.486 | 1.241 | 3.753 | 0.949 | 0.923 | 0.171 |
| | C21:0 | 0.656 | 0.981 | 0.417 | 0.311 | 1.601 | 0.534 |
| Total | | 55.763 | 65.097 | 59.623 | 79.010 | 61.506 | 56.174 |
| % of increase | | --- | (+)16.74 | (+)6.92 | (+)41.69 | (+)10.30 | (+)0.74 |
| | C14:1 | 6.571 | 3.471 | 7.365 | 7.284 | 4.694 | 7.981 |
| | C15:1 | 2.115 | 3.078 | 1.598 | 0.353 | 2.712 | 6.112 |
| | C16:1 | 2.023 | 2.344 | 1.828 | 1.114 | 1.764 | 2.007 |
| | C17:1 | 0.393 | 0.927 | 0.325 | 0.167 | 0.669 | 0.315 |
| | C18:1 | 16.066 | 10.086 | 12.500 | 3.799 | 9.345 | 7.970 |
| | C20:1 | 0.253 | -- | 0.273 | -- | 0.425 | 0.355 |
| | C22:1 | 2.371 | 1.503 | 1.595 | 0.429 | 3.183 | 4.003 |
| Total | | 29.79 | 21.409 | 25.484 | 13.146 | 22.792 | 28.743 |
| % of decrease | | --- | (-)28.13 | (-)14.45 | (-)56.14 | (-)23.49 | (-)3.51 |
| | C18:3 | 5.941 | 8.540 | 7.347 | 2.620 | 5.666 | 6.102 |
| | C18:2 | 7.785 | 8.400 | 6.802 | 3.217 | 7.533 | 8.475 |
| | C20:5 | 0.153 | -- | -- | 0.241 | 0.215 | -- |
| acids | C20:3 | -- | 0.287 | 0.353 | -- | 0.276 | 0.168 |
| | C20:2 | 0.276 | 1.007 | 0.244 | -- | 0.818 | -- |
| | C22:6 | 0.293 | -- | 0.154 | 0.228 | 1.194 | 0.223 |
| | C22:2 | -- | 0.254 | -- | 0.739 | -- | 0.111 |
| Total | | 14.448 | 18.488 | 14.9 | 7.045 | 15.702 | 15.079 |
| % of increase or decrease | | --- | (+)27.96 | (+)3.13 | (-)51.24 | (+)8.68 | (+)4.37 |
| Grand Total content | | 100.001 | 104.994 | 100.007 | 100.000 | 100.000 | 99.996 |
| % of increase or decrease | | --- | (+)4.993 | (+)0.006 | (-)-0.009 | (-)0.009 | (-)0.005 |

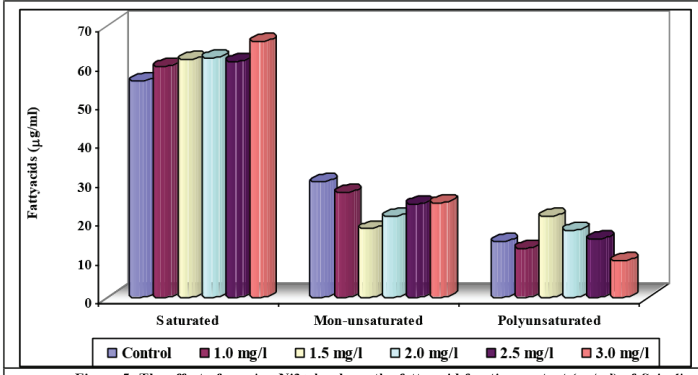


Figure 5: The effect of varying Ni2+ levels on the fatty acid fraction content (µg/ml) of *Spirulina platensis* grown for 10 days.

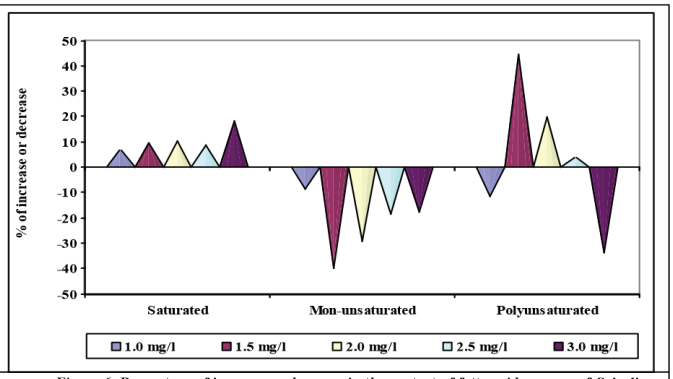


Figure 6: Percentage of increase or decrease in the content of fatty acids groups of *Spirulina platensis* cultured for 10 days under the effect of different Ni2+ concentrations compared to control.

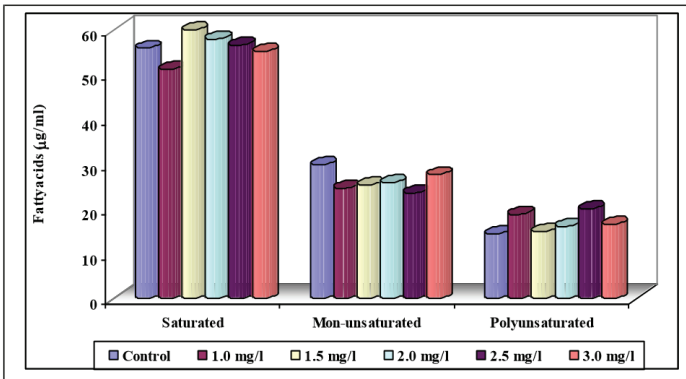


Figure 7: The effect of varying Cu2+ levels on the fatty acid fraction content (µg/ml) of *Spirulina platensis* grown for 10 days.

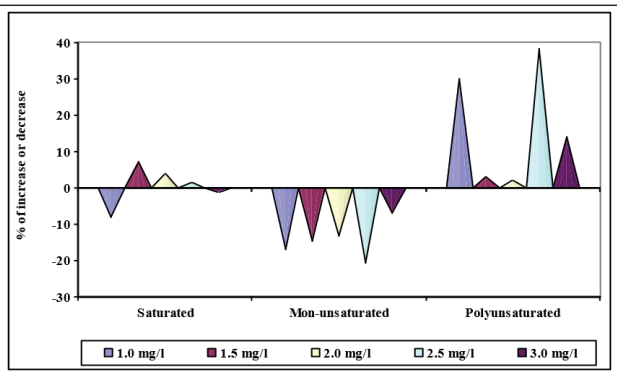


Figure 8: Percentage of increase or decrease in the content of fatty acids groups of *Spirulina platensis* cultured for 10 days under the effect of different Cu2+ concentrations compared to control.

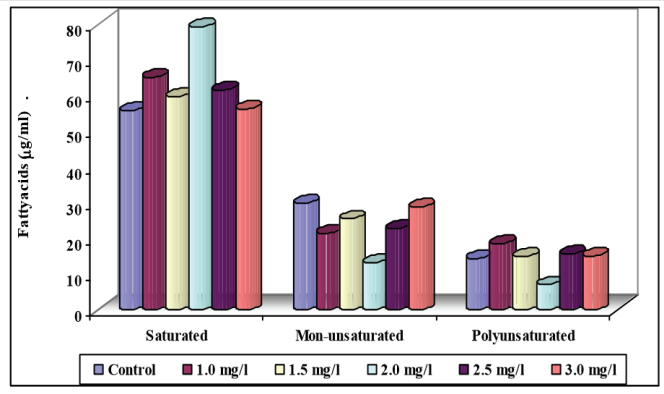


Figure 9: The effect of varying Zn2+ levels on the fatty acid fraction content (µg/ml) of *Spirulina platensis* grown for 10 days.

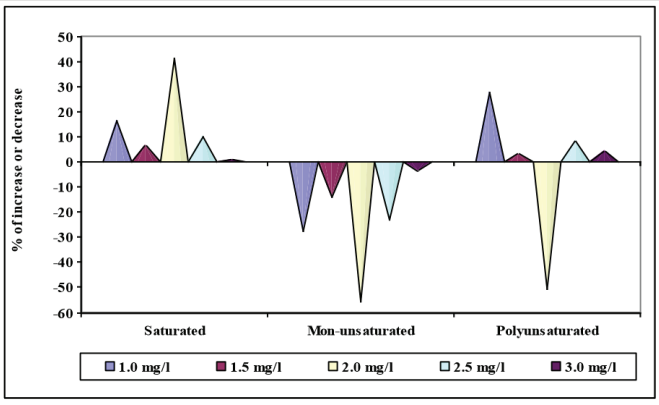


Figure 10: Percentage of increase or decrease in the content of fatty acids groups of *Spirulina platensis* cultured for 10 days under the effect of different Zn2+ concentrations compared to control.

## D- Protein profile:

The total soluble protein profile on the gel plate for the control and treated organisms at various concentrations of $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ (1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) revealed bands that were dispersed throughout the gel plate. There were 20 bands for $Zn^{2+}$ and 17 bands for both $Ni^{2+}$ and $Cu^{2+}$ that were observable on the gel plate and confirmed by scanning utilising the band peaks. Under the influence of different concentrations of the three tested elements ($Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$), some of these bands were common in both the control and the treated organism, while others were frequent only in the treated organism. Nearly all of the lanes saw the majority of them in the area between 25 KDa and 212 KDa. However, at all $Ni^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ concentrations, the number of these bands often increased as the element's concentration increased, (**Fig. 11- 13**). $Cu^{2+}$ stress had a greater impact than $Ni^{2+}$ and $Zn^{2+}$ stress on the protein profile in terms of the damaging effects of the heavy metal ions. These findings appeared to be consistent with those of several previous works (**Ahmed, 2010 ; El Taher, 2012 and El-Agawany and Kaamoush, 2022**).

However, the majority of the bands were visible in almost every lane between 25 KDa and 212 KDa. There were only 13 bands acquired for the control, whereas the number of bands varied depending on the kind and concentration of the material being tested. In the case of Ni, the sum of the bands increased as concentration of the element increased. At concentrations of 2.0, 2.5, and 3.0 mg/l Ni, the percent of increased bands

reached 30.8% over control for all three of the tested concentrations of Ni, whereas at concentrations of 1.0 and 1.5 mg/l Zn, the percent of increase in the total of bands was 15.4%. At concentrations of 1.0 and 1.5 mg/l Ni, there were 3 freshly formed bands; at values of 2.0, 2.5, and 3.0, there were 4 newly formed bands. This data makes it evident that the majority of freshly produced bands were found in low molecular weight regions, whereas the majority of bands that vanished were found in high molecular weight regions.

The acquired protein profile bands are scattered across the entire gel plate, as shown by these data. The majority of bands have cathodic anodic symmetry, while some bands are anodic and others cathodic. There were 20 bands for Zn and 17 bands for both Ni and Cu that were visible on the gel plate and confirmed by scanning utilising the band peaks. Under the influence of the five different concentrations of the three tested elements ($Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$), some of these bands were common in both the control and the treated organism, while others were frequent only in the treated organism. Another crucial statistic is that *Spirulina platensis* has an overall protein content of 82.63% in 50% wastewater effluent. It has been hypothesized that heavy metals in wastewater, even in small amounts, can speed up protein synthesis in *S. platensis*, (**Balaji *et al*., 2015**).

It is also evident that the newly generated bands increased in number from one to three when the Cu concentration was increased from 1.0 mg/l to 2.0 mg/l, two bands at 2.5 mg/l, and one band at 3.0 mg/l, respectively. Additionally, the total bands rose to 15 bands for Cu at concentrations of 1.0, 1.5, and 2.5 mg/l, and to 16 bands at 2.0 mg/l of Cu. At a concentration of 3.0 mg/l Cu, there were only 14 bands. In the instance of Zn, the number of bands grew as element concentration increased. At 2.0, 2.5, and 3.0 mg/l Zn, respectively, the newly generated bands rose by almost 5, 3, and 4 bands as the element concentration increased. Only one band vanished at Zn values of 2.0 and 2.5 mg/l. The sensitivity of *Spirulina platensis* to Cu, Zn, and eventually Ni ions may be explained by this map. **ElAgawany and Kaamoush (2022)** found that the toxicity of the zinc element affected the percentage growth in the number of bands in *Dunaliella tertiolecta* culture were verified. It is also apparent that the organism experienced severe harm as a result of the zinc element's toxicity at a dosage of 25 mg/L.

The protein profile of algae can also be impacted by various heavy metals. **Chernicova *et al*., (2006)** indicated that increasing manganese concentrations did not significantly alter cell ultrastructure or protein profile in *Spirulina platensis*. **Sinha and Hader, (1996)**, observed that *Anabaena* species cultured exhibited no changes in protein pattern under stress. On the other hand, **Fulda *et al*., (1999)** reported that Periplasmic proteins isolated from culture of *Synechocystis* species cultured under metal stress showed substantial changes in composition. **Hoyos and Zhang, (2000)** findings that coincide with the conclusions above) discovered that reversible protein phosphorylation/ dephosphorylation is crucial in signaling the plant's adaptive response to stress. The results of **Salah El-Din, (1994)** findings supported the idea that most algae species share physiological processes that are connected to the creation or breakdown of certain macromolecules. This finding appears to explain the various variations in total soluble protein band amounts in stressed algae.

**Ahmed, (2010) and El Taher, (2012)** noticed that the synthesis or accumulation of new proteins could be used to increase an organism's resistance to stress circumstances. These findings closely match those we obtained for *Spirulina platensis*. The majority of developing nations are very concerned about the lack of protein in human nutrition, hence new, unconventional protein sources must be developed. *Spirulina platensis* and other microalgal species, in particular, have a high protein concentration, which makes them a good source of this nutrient, (**Anne *et al*., 2016**).

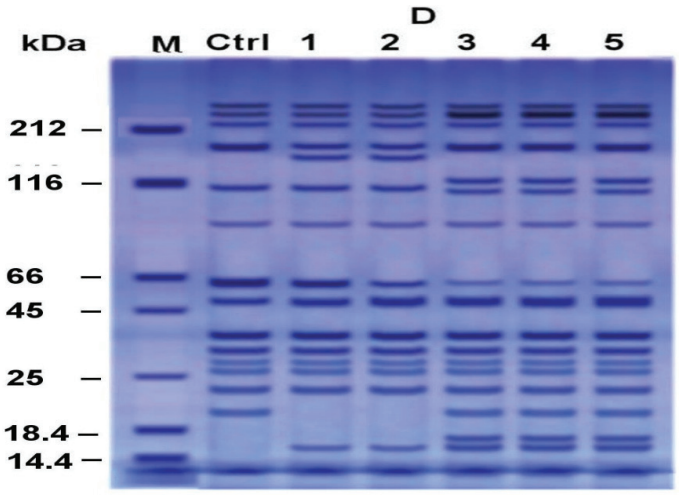


*Plate 1: Soluble protein profile bands pattern of the studied Spirulina platensis cells cultured for 10 days on control and under the effect of different Ni2+ concentrations. (Lane 1 (M) = marker; lane 2 (Ctrl) = control; lane 3 (1) = 1.0 mg/l Ni; lane 4 (2) = 1.5 mg/l Ni; lane 5 (3) = 2.0 mg/l Ni; lane 6 (4) = 2.5 mg/l Ni; lane 7 (5) = 3.0 mg/l Ni).*

*Table 4: Soluble protein profile pattern bands showing sum, unchanged, disappeared and newly formed bands at control and under the effect of different Ni2+ concentrations.*

| Treatment / Bands | Control | Different $Ni^{2+}$ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| Sum of bands | 13 | 15 | 15 | 17 | 17 | 17 |
| Unchanged bands | -- | 12 | 12 | 13 | 13 | 13 |
| Disappeared bands | -- | 1 | 1 | 0 | 0 | 0 |
| Newly formed bands | -- | 3 | 3 | 4 | 4 | 4 |

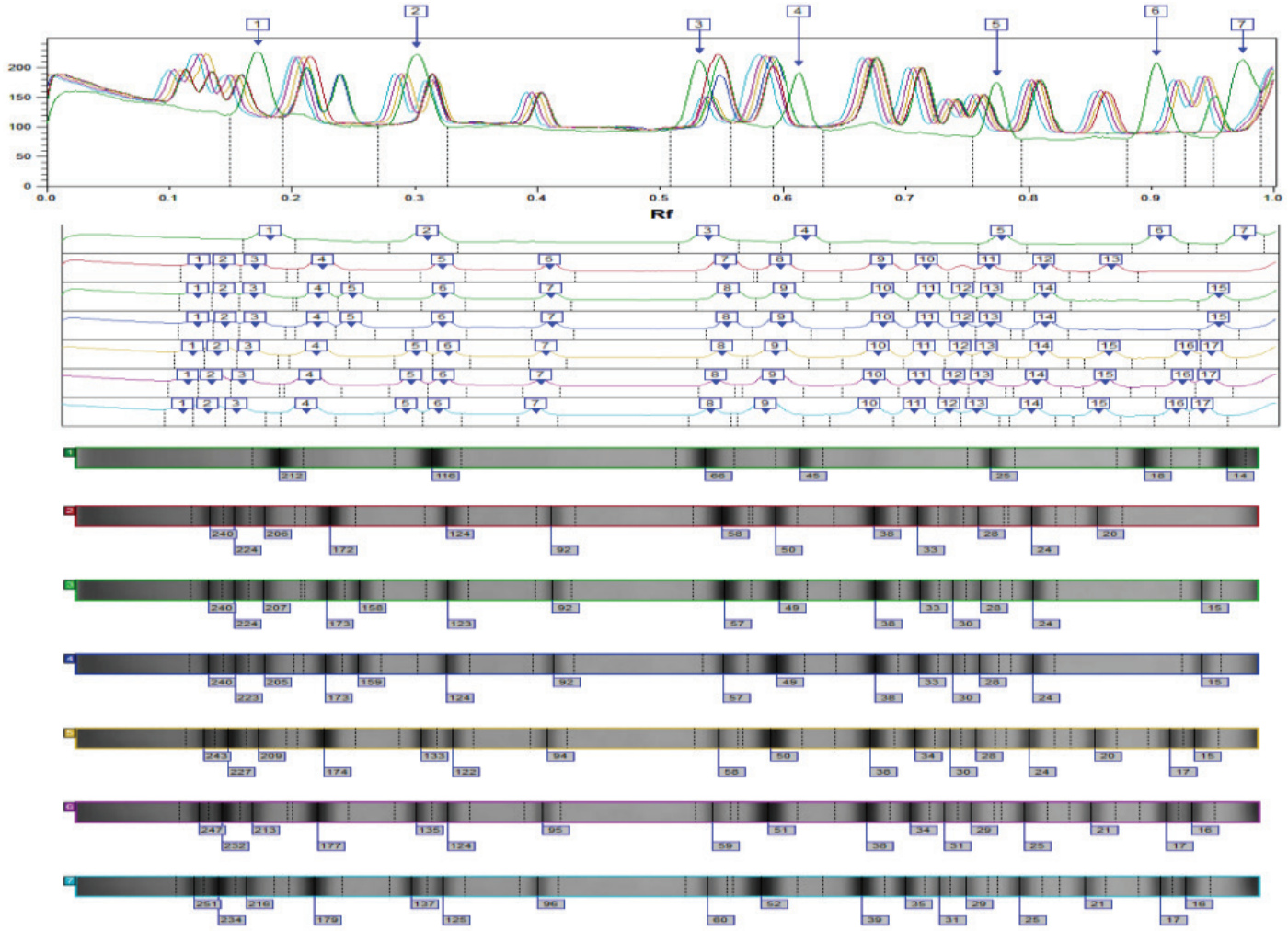


*Figure 11: Electropherogram showing the results of scanning of protein profile bands of Spirulina platensis cells cultured for 10 days on control and under the effect of different Ni2+ concentrations.*

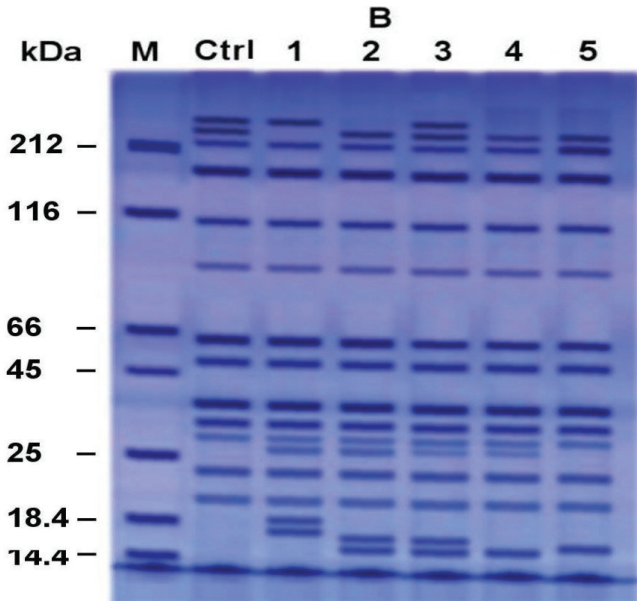


*Plate 2: Soluble protein profile bands pattern of the studied Spirulina platensis cells cultured for 10 days on control and under the effect of different Cu2+ concentrations. (Lane 1 (M) = marker; lane 2 (Ctrl) = control; lane 3 (1) = 1.0 mg/l Cu; lane 4 (2) = 1.5 mg/l Cu; lane 5 (3) = 2.0 mg/l Cu; lane 6 (4) = 2.5 mg/l Cu; lane 7 (5) = 3.0 mg/l Cu).*

*Table 5: Soluble protein profile pattern bands showing sum, unchanged, disappeared and newly formed bands at control and under the effect of different Cu2+ concentrations.*

| Treatment / Bands | Control | Different $Cu^{2+}$ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| Sum of bands | 13 | 15 | 15 | 16 | 15 | 14 |
| Unchanged bands | -- | 12 | 12 | 13 | 13 | 13 |
| Disappeared bands | -- | 1 | 1 | 0 | 0 | 0 |
| Newly formed bands | -- | 3 | 3 | 3 | 2 | 1 |

http://dx.doi.org/10.21622/MARLOG.2023.12.1.036
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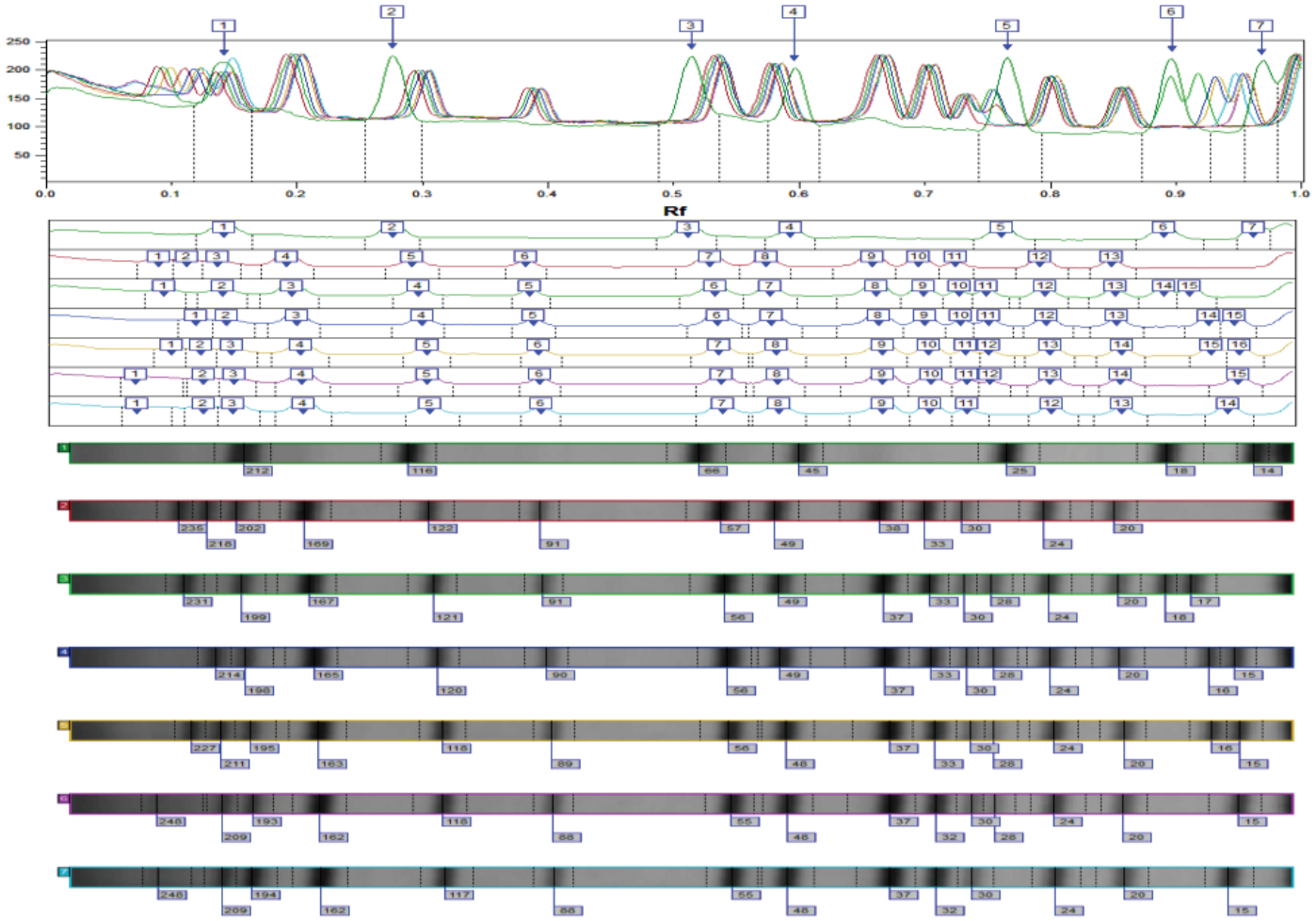


*Figure 12: Electropherogram showing the results of scanning of protein profile bands of Spirulina platensis cells cultured for 10 days on control and under the effect of different Cu2+ concentrations.*

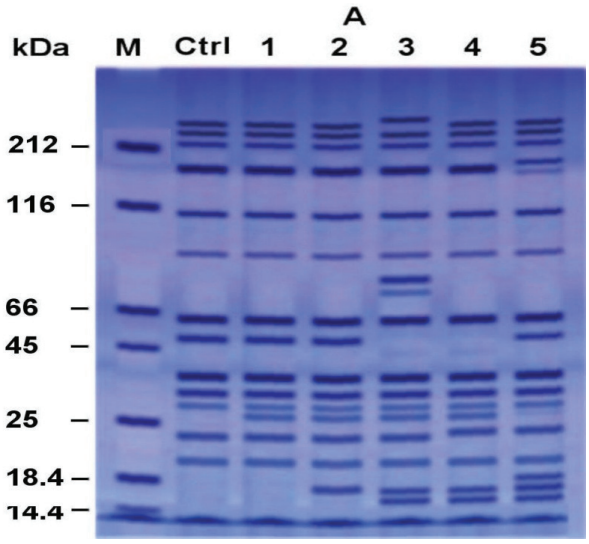


*Plate 3: Soluble protein profile bands pattern of the studied Spirulina platensis cells cultured for 10 days on control and under the effect of different Zn2+ concentrations. (Lane 1 (M) = marker; lane 2 (Ctrl) = control; lane 3 (1) = 1.0 mg/l Zn; lane 4 (2) = 1.5 mg/l Zn; lane 5 (3) = 2.0 mg/l Zn; lane 6 (4) = 2.5 mg/l Zn; lane 7 (5) = 3.0 mg/l Zn).*

*Table 6: Soluble protein profile pattern bands showing sum, unchanged, disappeared and newly formed bands at control and under the effect of different Zn2+ concentrations.*

| Treatment / Bands | Control | Different $Zn^{2+}$ concentrations (mg/l) | | | | |
|---|---|---|---|---|---|---|
| | | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| Sum of bands | 13 | 14 | 15 | 17 | 15 | 17 |
| Unchanged bands | -- | 13 | 13 | 12 | 12 | 13 |
| Disappeared bands | -- | 0 | 0 | 1 | 1 | 0 |
| Newly formed bands | -- | 1 | 2 | 5 | 3 | 4 |

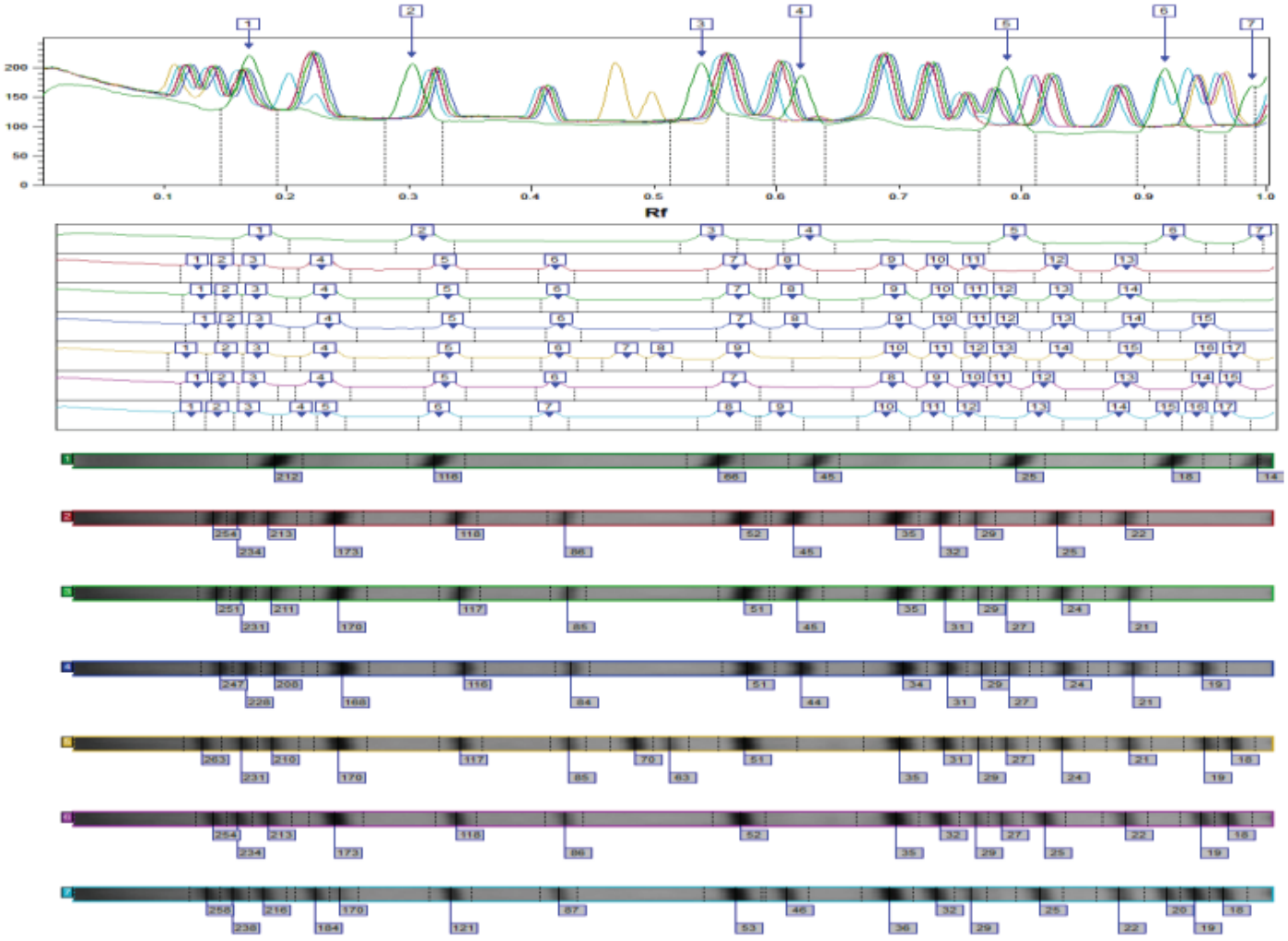


*Figure 13: Electropherogram showing the results of scanning of protein profile bands of Spirulina platensis cells cultured for 10 days on control and under the effect of different Zn2+ concentrations.*

## 3. CONCLUSION

Growth of *Spirulina platensis*, IR spectra, content of fatty acid fractions, and total soluble protein profile were all affected by the effects of various concentrations of $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$, and the results showed that the EC50 for all three heavy metals was almost recorded at a concentration of 2.0 mg/l. Optical density measurements showed that copper had a highest growth inhibitory impact than zinc or nickel did at all of the tested doses. IR spectra data indicated that novel compounds were generated in treated cells as opposed to untreated cells, and as a result, new peaks in the spectra developed while other compounds vanished. Compared to $Zn^{2+}$ and $Ni^{2+}$, this was clearer in the case of $Cu^{2+}$. Harmful effects of the three elements changed depending on the type of fatty acid group. As a consequence, the overall amount of the three fatty acids groups was decreased as the element's concentration was raised. Total fatty acids decreased under stress at all concentrations examined, and saturated fatty acids outnumbered unsaturated fatty acids. $Cu^{2+}$ stress was more significant than $Zn^{2+}$ and $Ni^{2+}$ stress in terms of the detrimental effect of metal ions on protein profile. The production or accumulating of new proteins may increase an organism's resistance to stressful situations. The sequence of toxicity of the three heavy metals ions is $Cu^{2+}$ in the first order, followed by $Zn^{2+}$ and $Ni^{2+}$ at all examined concentrations, indicating that $Cu^{2+}$ is more toxic than $Zn^{2+}$ and $Ni^{2+}$ metal ions and that the degree of stress is primarily influenced by the concentration and type of element as well as the duration of the culture period. The majority of developing countries are very concerned about protein deficit in human nutrition, hence new unorthodox protein sources must be created. Different microalgal species, especially *Spirulina platensis*, have a high protein content, which makes them ideal sources of this nutrient.

**Ethical Approval:** Not Applied
**Consent to Participate:** I freely consent to take part in this research study.

I understand that even if I accept to participate now, I have the right to withdraw at any moment or choose not to answer any question without consequence.

**Consent to Publish:** The authors warrant that the work has not been previously published in any form and is not being considered by another publisher, that the above-mentioned individuals are listed in the correct order, that no author entitled to credit has been omitted, and that the authors have the right to make the grants made to the Publisher complete and unencumbered.

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**Authors** Contributions: N.I.A., M.I.A.K. and H.E. contributed equally to the manuscript preparation. N.I.A., M.I.A.K. and H.E. designed the experiment and performed the laboratory analyses. N.I.A., M.I.A.K. and H.E. carried out the statistical analyses and tabulated the study results.

N.I.A., M.I.A.K. and H.E. wrote the first draft and revised the final version of the paper. N.I.A.,

M.I.A.K. and H.E. read and agreed on the submitted paper.

**Funding:** No funding was received.

**Competing Interests:** he authors state that they do not have any competing interests.

**Availability of data and materials:** This article includes and makes accessible all of the data produced or analysed during this investigation.

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