MIKAILALSYS
Journal of Multidisciplinary Sciences

e-ISSN : 2987-2286
p-ISSN : 2987-3924

Index : Dimensions, Scilit, Crossref, Garuda, Semantic, Lens,Google Scholar,Base, etc

https://doi.org/10.58578/mikailalsys.v3i2.6116

Effects of Cefuroxime and Varying Nitrogen Conditions on Growth and Pigment Composition of *Chlorella* sp.

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Article Info:

Submitted:	Revised:	Accepted:	Published:
Apr 26, 2025	May 23, 2025	Jun 3, 2025	Jun 8, 2025

Abstract

This study addresses the limited research on the ecological impacts of cefuroxime on freshwater microalgae, despite the antibiotic's widespread presence in aquatic environments. Specifically, it investigates the effects of cefuroxime on the growth and pigment composition, measured through cell density, chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, and carotenoids of *Chlorella* sp. under nitrogen-replete and nitrogen-limited conditions. A quantitative experimental design was applied, comprising five treatment groups cultured in triplicate. Data collection involved microscopic cell counts and spectrophotometric analysis, while growth and pigment dynamics were assessed using specific growth rate equations and pigment concentration formulas. Statistical analyses included the Shapiro-Wilk test, Levene's test, repeated measures ANOVA, and Duncan's Multiple Range Test ($p \leq 0.05$). Results indicated that cefuroxime initially stimulated growth and pigment production under nitrogen-replete conditions but led to significant inhibition over time. Conversely, the control group demonstrated the most stable growth and pigment accumulation by day seven. These outcomes challenge the notion that low-dose antibiotics are ecologically benign and underscore the critical role of nitrogen availability in modulating antibiotic effects. The study concludes that cefuroxime exerts time- and nutrient-dependent impacts on *Chlorella* sp., advancing ecotoxicological theory by elucidating the interactive effects of nutrient stress and pharmaceutical contamination. Practical implications highlight the necessity for stricter regulation of pharmaceutical effluents and improved nutrient management in aquatic systems. Future investigations are recommended to explore the combined effects of multiple antibiotics under varied nutrient regimes.

Keywords: Cefuroxime; Nitrogen Limitation; *Chlorella* sp.; Pigment Composition; Aquatic Ecotoxicology

INTRODUCTION

Microalgae, particularly *Chlorella* sp., are pivotal primary producers in aquatic ecosystems, playing a critical role in nutrient cycling and energy transfer (Graham et al., 2021; Circuncisão et al., 2022). Their ability to thrive in diverse environmental conditions, including polluted waters, makes them ideal candidates for studying the ecological impacts of contaminants such as pharmaceuticals (Ugya et al., 2024). Among these contaminants, antibiotics like cefuroxime have emerged as significant pollutants due to their widespread use in human and veterinary medicine and their persistence in aquatic environments (Das et al., 2019 and Ewa et al., 2020).

Cefuroxime, a second-generation cephalosporin, is frequently detected in wastewater and surface water due to incomplete metabolization and disposal (Ribeiro et al., 2018 and Lenart-Boroń et al., 2020). Its ecological impact is of growing concern, as it can disrupt microbial communities and alter the growth dynamics of primary producers like *Chlorella* sp. (Das et al., 2019). Microalgae are particularly vulnerable to antibiotic stress, which can impair their photosynthetic efficiency, biomass production, and nutrient uptake (Gonzalez-Pleiter et al., 2013; Xu et al., 2019a). Understanding these effects is essential for assessing the broader ecological consequences of antibiotic pollution.

Nitrogen availability is another critical factor influencing microalgal growth. Under nitrogen-limited conditions, microalgae exhibit reduced growth rates and altered metabolic pathways, while replete nitrogen conditions support optimal biomass production (Yaakob et al., 2021). The interaction between nitrogen availability and Cefuroxime stress remains poorly understood, particularly for species like *Chlorella* sp., which are often used in bioremediation and biofuel production. This study aims to bridge this gap by investigating how cefuroxime affects *Chlorella* sp. under varying nitrogen conditions.

The contamination of aquatic ecosystems with pharmaceuticals, particularly antibiotics like cefuroxime, has become a pressing environmental issue (Carvalho & Santos, 2016 & Prateek et al., 2022). The widespread use of cefuroxime in medical and agricultural practices has led to its accumulation in water bodies, where it can adversely affect non-target organisms, including microalgae (Ribeiro, 2017 & Yu et al. 2022). Microalgae, as foundational components of aquatic food webs, are highly sensitive to environmental changes, making them reliable indicators of ecosystem health (Lemley et al., 2016 & Wu et al., 2017). However, the specific effects of cefuroxime on *Chlorella* sp. under different nitrogen regimes remain unexplored.

Nitrogen is a key nutrient that regulates microalgal growth and productivity. Its scarcity or abundance can significantly alter physiological responses, including pigment synthesis and cell division (Benavente-Valdés et al., 2016; Li et al., 2019 & Tarazona et al., 2021). The combined stress of nitrogen limitation and antibiotic exposure could exacerbate negative effects on microalgae, potentially disrupting aquatic ecosystems. This study seeks to address these concerns by evaluating the growth and physiological responses of *Chlorella* sp. to cefuroxime under low and replete nitrogen conditions.

The increasing detection of cefuroxime in aquatic environments underscores the need for comprehensive studies on its ecological impacts (Wang et al., 2018). Microalgae, such as *Chlorella* sp., are not only vital for ecosystem functioning but also serve as model organisms for ecotoxicological research (Xu et al., 2019). By examining the interaction between cefuroxime and nitrogen availability, this study will provide critical insights into the resilience of microalgae to combined stressors.

In Nigeria, where eutrophication and pharmaceutical pollution are growing concerns, understanding these interactions is particularly relevant (Chalew and Halden, 2009; Kayode - Afolayan et al., 2022; Alexander & Imegi, 2024). The findings of this study will contribute to the development of strategies for mitigating the impacts of antibiotic pollution and nutrient imbalances in aquatic systems. Additionally, the results will offer baseline data for future research on microalgal responses to emerging contaminants.

The aim of this study is to evaluate the effects of cefuroxime on the growth and physiological parameters (cell density, chlorophyll – a, chlorophyll – b, total chlorophyll, and carotenoid contents productions) of *Chlorella* sp. under low and replete nitrogen conditions.

METHODS

The *Chlorella* sp. strain was obtained and maintained in the Phycology Laboratory, Department of Botany Ahmadu Bello University, Zaria. Strains were maintained in the same Laboratory. Experimental cultures were maintained in Blue Green 11 (BG 11) medium (pH 7.4) under controlled laboratory conditions (light intensity 40µmol $m^{-2}s^{-1}$, photoperiod 16:8 h light:dark and temperature of 23±1°C). The BG-11 medium, used for culturing the *Chlorella* sp., consists of several macro- and micronutrients dissolved in distilled water. The formulation per liter includes $NaNO_3$ (30.00 g), K_2HPO_4 (8.00 g), $MgSO_4 \cdot 7H_2O$ (15.00 g), $CaCl_2 \cdot 2H_2O$ (7.20 g), $C_6H_8O_7.1H_2O$ (1.20 g), $C_{12}H_8FeN_3O_{14}$ (1.20 g), Na_2EDTA (0.20 g), and Na_2CO_3 (4.00 g). In addition, a trace metal mix was added at 1.0 mL per liter. This trace metal solution is prepared in 100 mL of distilled water and includes H_3BO_3 (1.430 g), $MnCl_2 \cdot 4H_2O$ (0.905 g), $ZnSO_4 \cdot 7H_2O$ (0.110 g), $Co(NO_3)_2 \cdot 6H_2O$ (0.025 g), $CuSO_4 \cdot 5H_2O$ (0.040 g), and $Na_2MoO_4 \cdot 2H_2O$ (0.195 g). Sodium (Na) was supplied by $NaNO_3$ in this medium. The BG 11 medium was sterilized by autoclaving (121°C, 30 min) 24 hours before use. The organisms' acclimation involved consecutive culture transfer at exponential growth phase and each new culture was monitored for its growth rate to obtain reliable physiological results. Experimental conditions were kept constant throughout the experiment.

The toxicity test was carried out according to the guideline of the Organization for Economic Cooperation and Development (OECD, 2011) for testing chemicals no. 201 with modifications. The experiment was carried out in 250mL Erlenmeyer flasks with 100mL of the algae in the exponential growth phase.

In the main test, *Chlorella* sp. was exposed to cefuroxime in limited and replete nitrogen for the incubation of 7 days. The experimental setup was as follows; the Control which is made up of BG – 11 media only, Limited Nitrogen (LN – Nitrogen in excluded from the media), Limited Nitrogen plus 0.05 mg/L of cefuroxime (LN+0.05), Replete Nitrogen plus 0.05 mg/L of cefuroxime (RN+0.05) – normal media with full nitrogen contents and the antibiotics.

All cultures were cultivated according to the condition described above, for seven days. The initial biovolume of the microalgae was $4.0\times10^{6}\mu M^{3}mL^{-1}$. All experimental treatments were carried out in triplicates. Aliquots were collected daily during the experiments for cell density and pigment content composition. All operations were carried out under sterile conditions to avoid contamination.

Biovolume and morphological examination were carried out at the beginning of the experiment. Cell density was determined microscopically by cell counts using an improved bright lined Neubauer haemocytometer. Optical density of the cultures was measured daily as absorbance (750nm) using a UV-VIS spectrophotometer (Spectrumlab 722N). A combination of microscopic counts and absorbance readings were used to monitor growth following calibration curves. Cell dimension and biovolumes were calculated according to Hillebrand *et al.* (1999). Specific growth rate (μ) was determined according to the equations by Chia *et al.* (2013).

$$\mu = \text{Log}\,\frac{\text{ABS (t2)}}{(a\times \text{ABS (t1)})} \times (t2-t1)^{-1} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

$$a = \frac{T_{vol} - R_{vol}}{T_{vol}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Where,

ABS = absorbance at 684nm

t = time

T_{vol} = total volume

R_{vol} = removed/replaced volume

Chlorophyll extraction was done using 80% (v/v) acetone (Shoaf and Lium, 1976) and absorbance was read at 470nm, 653nm and 666nm using a UV-VIS spectrophotometer. The concentration of the pigment was determined using the equation provided by Ritchie (2006).

Equation…..1: $\text{Chl a } (\mu g\ L^{-1}) = 17.12.\ A_{666} - 8.68.\ A_{653}$

Equation…..2: $\text{Chl b } (\mu g\ L^{-1}) = 32.23.\ A_{653} - 14.55.\ A_{666}$

Equation…..3: $\text{Total Chl } (\mu g\ L^{-1}) = 2.57.\ A_{666} + 23.6.\ A_{653}$

Levene’s homogeneity of variance and Shapiro-wink test were used to test for homogeneity and normality of the data respectively. Repeated measure Analysis of Variance (ANOVA) was used to determine significant variations in growth and photosynthetic pigments. Duncan's Multiple Range Test (DMRT) was used to separate the means where significant difference exists ($p \leq 0.05$). R. Statistics v. 4.3.1 for windows was used

RESULTS

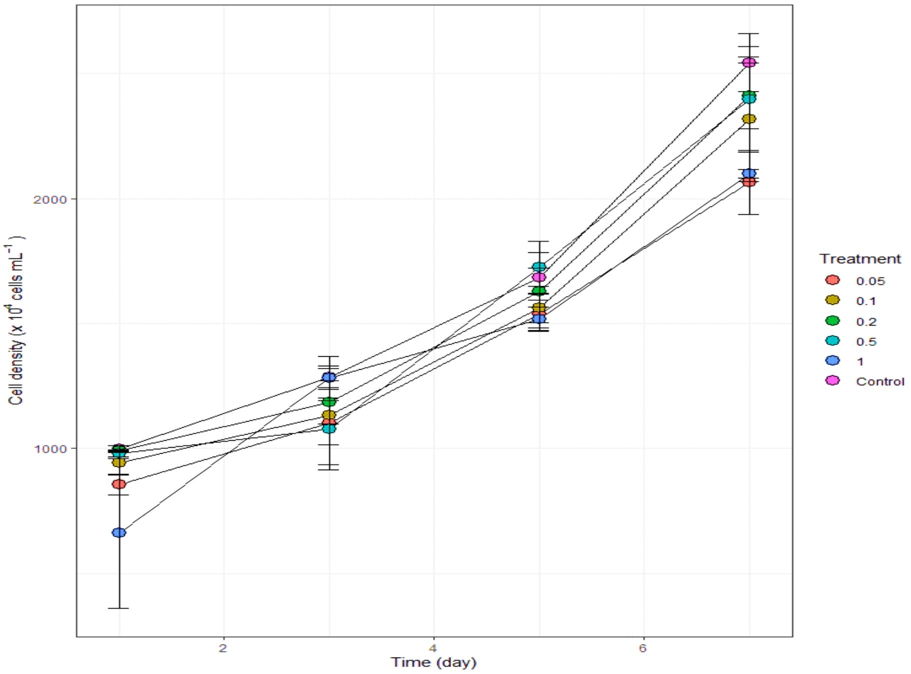

Fig. 1. *Chlorella* sp. Cell density as a function of Cefuroxime treatments under low and replete nitrogen

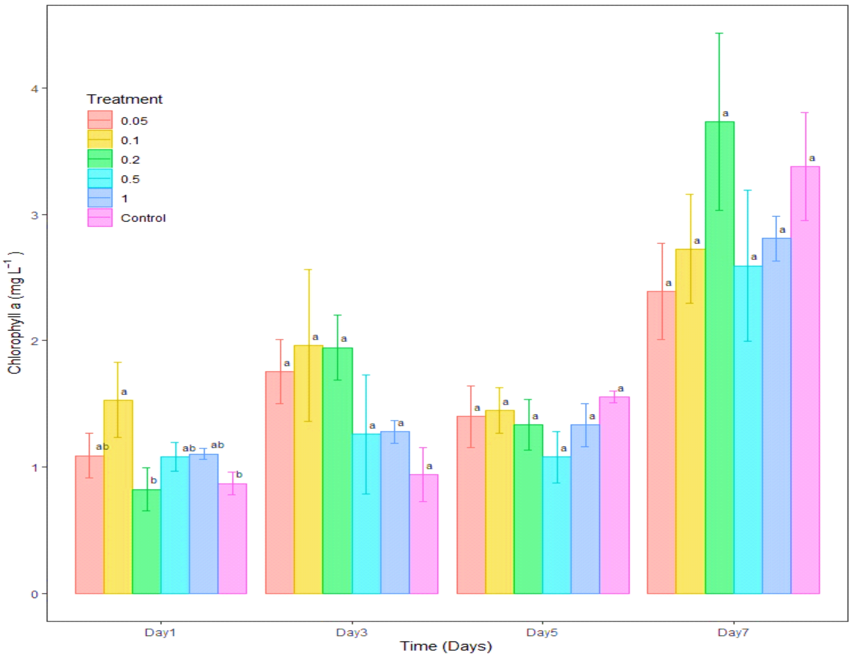

Fig. 2. Chlorophyll a content of *Chlorella* sp. exposed to Cefuroxime under varying nitrogen conditions and time

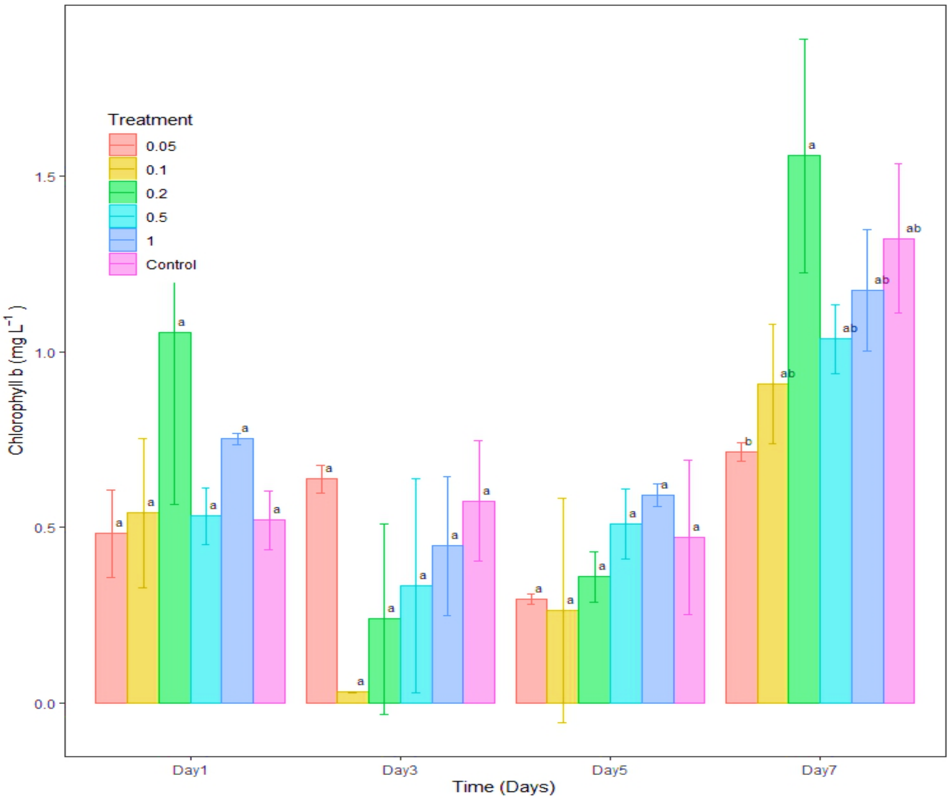

Fig. 3. Chlorophyll b content of Chlorella sp. exposed to Cefuroxime under varying nitrogen conditions and time

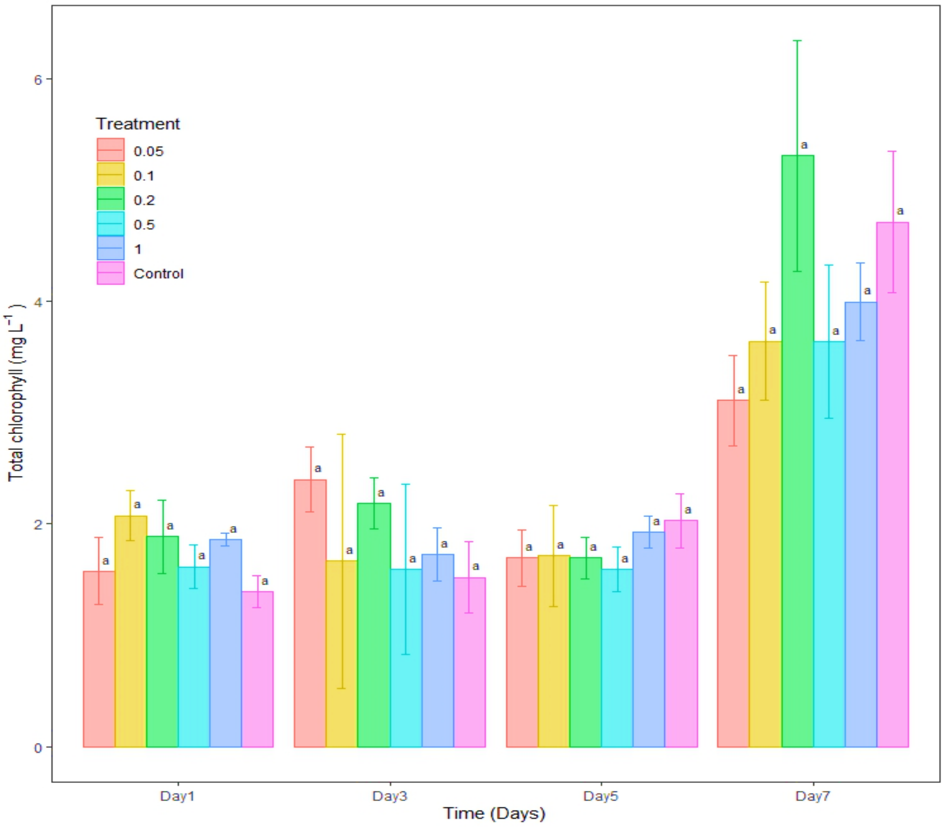

Fig. 4. Total chlorophyll content of Chlorella sp. exposed to Cefuroxime under varying nitrogen conditions and time

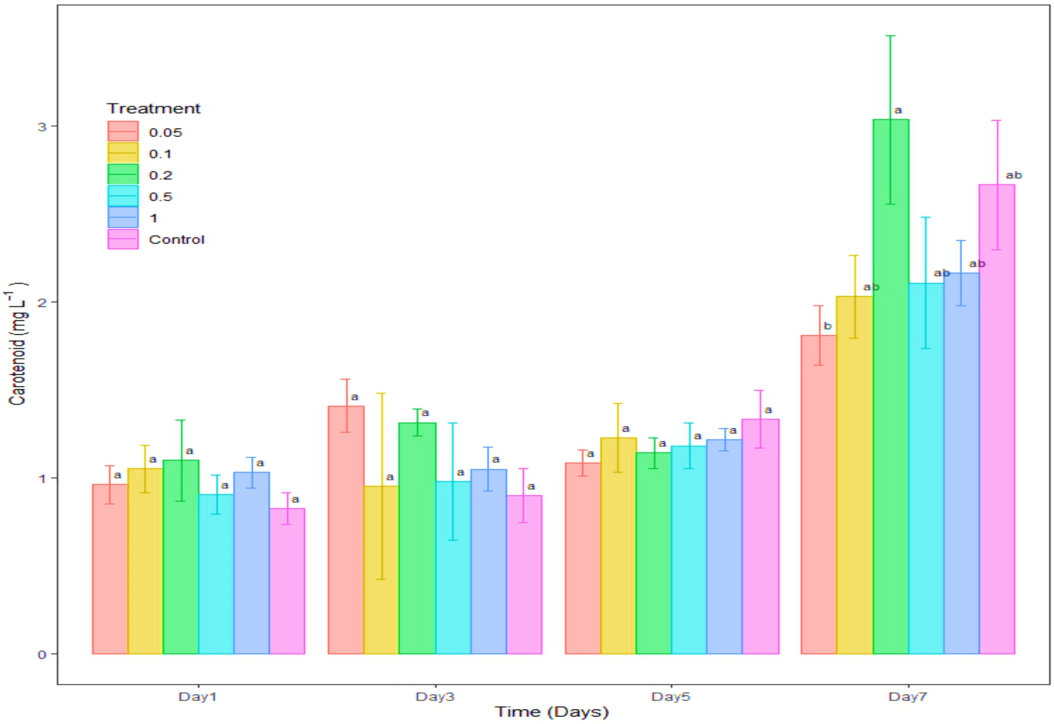

Fig. 5. Carotenoid content of Chlorella sp. exposed to Cefuroxime under varying nitrogen conditions and time

The study investigated the effects of cefuroxime on *Chlorella* sp. under different nitrogen conditions, measuring cell density, chlorophyll - a, chlorophyll - b, total chlorophyll, and carotenoid content over seven days. Initially, all treatments—Control, LN (limited nitrogen), LN+0.05 (limited nitrogen with cefuroxime), and RN+0.05 (replete nitrogen with cefuroxime)—started with similar cell densities (as shown in fig. 1) around 1000 × 10^4 cells mL^{-1}. By Day 3, the RN+0.05 treatment showed a sharp increase in cell density, exceeding 2000 × 10^4 cells mL^{-1}, suggesting that cefuroxime under nitrogen-rich conditions initially stimulated rapid growth. However, this effect was short-lived, as cell density stabilized and then declined slightly by Day 5. In contrast, the Control and LN treatments exhibited more gradual growth, with the Control ultimately achieving the highest cell density by Day 7. This indicates that while cefuroxime may trigger an early growth response in nitrogen-rich environments, untreated conditions provide more stable and sustained growth over time.

Chlorophyll - a levels revealed distinct patterns across treatments as seen in fig. 2. On Day 1, LN and LN+0.05 had significantly higher chlorophyll - a than the Control and

RN+0.05, suggesting that nitrogen limitation alone or with cefuroxime initially promoted pigment production. By Day 3, LN maintained the highest levels, while RN+0.05 remained intermediate, hinting that cefuroxime in nitrogen-rich conditions might slightly suppress chlorophyll - a. A notable shift occurred by Day 5, where RN+0.05 exhibited the lowest chlorophyll - a, indicating that prolonged exposure to cefuroxime under replete nitrogen significantly inhibited pigment synthesis. By the end of the experiment, the Control group surpassed all treatments in chlorophyll - a content, reinforcing that stress-free conditions favor optimal pigment accumulation.

As presented in fig. 3, Chlorophyll - b dynamics followed a similar yet distinct trajectory. On Day 1, LN had the highest levels, followed by LN+0.05 and RN+0.05, while the Control lagged behind. This suggests that nitrogen limitation, with or without cefuroxime, initially enhanced chlorophyll - b production. By Day 3, differences between treatments diminished, but by Day 5, LN+0.05 and RN+0.05 showed elevated chlorophyll - b compared to the Control, though statistical significance was marginal. By Day 7, the Control group exhibited the highest chlorophyll - b levels, significantly exceeding the LN treatment, while LN+0.05 and RN+0.05 occupied an intermediate position. This implies that while cefuroxime treatments initially influenced chlorophyll - b, the Control eventually outperformed them, further emphasizing the long-term benefits of untreated conditions.

Total chlorophyll content emulated these trends, with LN and LN+0.05 leading on Day 1, followed by a convergence of all treatments by Day 5. However, by Day 7, the Control group displayed a dramatic surge in total chlorophyll, far exceeding the treated groups. This suggests that while cefuroxime and nitrogen variations caused transient fluctuations in pigment production, the absence of stressors allowed for the most robust and sustained chlorophyll synthesis.

Carotenoid content also exhibited treatment-specific responses. Initially, LN and LN+0.05 had the highest carotenoid levels, while Control and RN+0.05 were significantly lower. By Day 3, all treated groups showed increased carotenoids, though the Control remained comparatively low. By Day 5, differences between treatments disappeared, but by Day 7, the Control group experienced a substantial increase, surpassing all cefuroxime-exposed treatments. This indicates that carotenoid production, like chlorophyll, is initially influenced by nitrogen availability and cefuroxime but ultimately thrives best in untreated conditions.

In summary, the study highlights that cefuroxime's effects on *Chlorella* sp. depend heavily on nitrogen availability and exposure duration. While certain treatments (e.g., RN+0.05) induced rapid early growth or pigment production, these effects were often unsustainable. In contrast, the Control group consistently demonstrated superior performance by Day 7 in all measured parameters, underscoring the importance of stress-free conditions for long-term algal health and productivity. These findings suggest that while cefuroxime and nitrogen manipulation can transiently alter microalgal physiology, untreated environments provide the most stable and favorable conditions for sustained growth and pigment accumulation.

DISCUSSION

The study observed significant variations in cell density across treatments over seven days. Initially, all groups (Control, LN, LN+0.05, RN+0.05) had similar cell densities. By day 3, the RN+0.05 treatment (replete nitrogen + cefuroxime) showed a sharp increase, suggesting a stimulatory effect of cefuroxime under nitrogen-rich conditions. However, this effect plateaued by day 5, possibly due to antibiotic-induced stress or nutrient depletion. In contrast, the Control group exhibited the highest cell density by day 7, indicating that untreated conditions favor sustained growth. The early growth surge in RN+0.05 may result from cefuroxime acting as a carbon source or hormesis effect – where low antibiotic doses may transiently stimulate growth. However, long-term exposure likely caused oxidative stress or metabolic disruption, aligning with studies of Li et al. (2022) on antibiotics, and Huang (2024) on cephalosporins, inhibiting microalgal growth. Similarly, Amirian & Movafeghi (2025) reported that, early growth stimulation was followed by growth inhibition in their studies on *Chlorella vulgaris* exposed to cephalexin. The Control's stability contrasts with findings in *Chlorella vulgaris* exposed to antibiotics by González-Pleiter et al., (2013), where growth inhibition was dose-dependent. Likewise, studies by Guo et al. (2015) and Yu et al. (2017) showed findings for ceftazidime in green algae. Here, cefuroxime's impact was transient, suggesting species-specific tolerance. Unlike other β-lactams (e.g., penicillin), cephalosporins like cefuroxime may exhibit biphasic effects due to their broader-spectrum activity (Ribeiro et al., 2018).

Chlorophyll – a was initially higher in LN (low nitrogen) and LN+0.05 treatments, indicating nitrogen limitation which has been reported to promotes chlorophyll pigment synthesis as an adaptive response (Truong et al., 2024). This action obviously led to the higher productions of the chlorophyll – a content in the treatments concerned. However, by day 7, the Control surpassed other groups, while cefuroxime-exposed treatments (especially RN+0.05) showed suppression. The decline in RN+0.05 suggests cefuroxime disrupts chlorophyll biosynthesis, similar to erythromycin's effect on Scenedesmus (Wang et al., 2021) and cephlaxin's impact on *Chlorella vulgaris* (Amirian et al., 2025). The late-stage dominance observed in the Control, implies that untreated cells recover pigment synthesis, whereas cefuroxime causes irreversible damage or reacts with photosynthetic enzymes. Yu et al. (2017) observed a similar irreversible pigment loss when green algae were exposed to ceftazidime. While Ribeiro et al., (2018) affirms that cephalosporins generally inhibit pigment synthesis more severely than macrolides (e.g., erythromycin), likely due to their higher reactivity with photosynthetic enzymes.

Chlorophyll – b trends mirrored chlorophyll – a, with LN treatments initially higher just as it were in *Chlorella sorokiniana* under nitrogen stress as reported by Negi et ai., (2022). By day 7, the Control group led, while LN+0.05 and RN+0.05 showed intermediate levels. This stimulation however, contrasts with its overall inhibitory trend, possibly due to transient upregulation of stress proteins. Similar biphasic responses were noted in Chlamydomonas under antibiotic stress by Yildirim (2025) where stress-response pathways temporarily upregulated pigment production. A notable, finding here is that unlike tetracyclines, which degrade chlorophyll – b preferentially (Rydzyński et al., 2019), cephalosporins like cefuroxime affect both chlorophyll types equally.

Total chlorophyll was lowest in the Control on day 1 but peaked by day 7. LN treatments had an early surge of total chlorophyll content production. This initial advantage in LN reflects nitrogen scarcity triggering pigment accumulation as recorded by Wang et al., (2021) in *Scenedesmus* under nitrogen scarcity. The replete plus cefuroxime treatment (RN+0.05) expressed suppression of the synthesis of total chlorophyll content production. This suppressive action of (RN+0.05) suggests an interference with nitrogen assimilation pathways in the cells of the organism, as seen with sulfonamides in Anabaena (Zhong et al., 2021). In a similar work by Hutárová et al., (2022), it was observed that cefixime inhibited nitrogenase in cyanobacteria, thus, suggesting a cross-talk between antibiotic stress and nitrogen metabolism, which corresponds to the actions of cefuroxime's suppression in RN+0.05. This assertion is reinforced by the findings of Ansari et al., (2025) and Guo &

Chen (2016) who stated that Cephalosporins disrupt total chlorophyll more persistently than sulfonamides, which often show reversible effects.

Carotenoids were highest in LN groups early, likely as antioxidants under stress. This finding matches with oxidative stress responses in *Chlorella* exposed to ceftazidime as described by Yu et al., (2017). By day 7 of the exposure, the Control surged, while cefuroxime treatments plateaued. The late surge in the Control contrasting with cefuroxime's plateau, is akin to fluoroquinolones in Microcystis (Pauletto & De Liguoro, 2024)), and cefotaxime's suppression of antioxidant enzymes in *Microcystis* as reported by Wang et al., (2023). This can be further validated by the works of Das et al., (2029) which states that Cephalosporins (e.g., cefuroxime) deplete carotenoids more rapidly than fluoroquinolones, likely due to stronger ROS generation.

CONCLUSION

The study examined how cefuroxime affects various growth parameters of Chlorella sp. under different nitrogen conditions over seven days. Regarding cell density, cefuroxime showed contrasting effects depending on nitrogen availability. In nitrogen-replete conditions (RN+0.05), the antibiotic initially stimulated rapid cell proliferation by day 3, likely through a hormetic effect or by serving as an additional carbon source. However, this growth surge proved temporary, with cell densities leveling off by day 5 and ultimately being surpassed by the control group by day 7. This pattern suggests that while cefuroxime may provide short-term growth benefits under nutrient-rich conditions, prolonged exposure becomes inhibitory, possibly due to metabolic stress or resource depletion. In nitrogen-limited treatments (LN+0.05), cell growth followed a more gradual trajectory, never exceeding the final density achieved by the untreated control, indicating that antibiotic stress compounds the challenges of nitrogen limitation.

The pigment analysis revealed consistent patterns of cefuroxime interference with photosynthetic systems. Chlorophyll a production initially responded positively to nitrogen limitation in LN treatments, consistent with known stress responses in microalgae. However, by the experiment's conclusion, the control group exhibited superior chlorophyll a accumulation, while the RN+0.05 treatment showed significant suppression. This demonstrates cefuroxime's capacity to disrupt chlorophyll synthesis, particularly in nitrogen-replete environments where the antibiotic's effects appear most pronounced.

Chlorophyll b dynamics mirrored this pattern, though with less dramatic differences between treatments, suggesting cefuroxime affects both chlorophyll types similarly rather than targeting one preferentially. The total chlorophyll results reinforced these observations, with early advantages in nitrogen-limited treatments giving way to control group dominance by day 7, while the RN+0.05 group consistently underperformed in pigment production.

Carotenoid content followed a somewhat different but related trajectory. Nitrogen-limited treatments initially showed elevated carotenoid levels, likely reflecting their role as antioxidant molecules in stressed cells. However, the sustained increase in the control group contrasted sharply with the plateau observed in cefuroxime-treated samples, indicating that while stress conditions may trigger temporary antioxidant responses, they cannot match the steady pigment accumulation occurring in unstressed cells. This pattern suggests that cefuroxime exposure, particularly in nitrogen-replete conditions, may overwhelm the algae's antioxidant defenses over time, leading to pigment degradation or impaired synthesis.

The collective findings demonstrate that cefuroxime's effects on Chlorella sp. are complex and context-dependent. While the antibiotic can produce transient growth stimulation or stress responses, particularly under nitrogen-replete conditions, these effects invariably give way to inhibition over time. The control group's consistent superiority in both growth and pigment parameters by the experiment's conclusion underscores the fundamentally stressful nature of antibiotic exposure for microalgae. These results have important implications for understanding how cephalosporin antibiotics may impact primary producers in aquatic ecosystems, particularly in environments with varying nutrient availability. The findings suggest that even sub-lethal antibiotic concentrations can significantly alter algal physiology and productivity, with potential consequences for ecosystem function and biogeochemical cycles.

Acknowledgements

The research was conducted with support from Tertiary Education Trust Fund (TETFUND) Institutional Based Research (IBR) Grant, 2023 cycle.

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