Study of the green microalga *Chlorella sorokiniana* for the removal of nutrients and of multiple antibiotics in wastewater treatment

Abstract: Obtaining clean water is a global priority as emphasized by the United Nations Sustainable Development Goal 6, which aims to ensure availability and sustainable management of water and sanitation for all. Pharmaceutical pollutants are becoming more prevalent in aquatic environments, triggering public health concerns, negative environmental impacts, and the development of antibiotic resistance. Microalgae hold great potential for bioremediation of antibiotics, although most of the studies to date supporting these observations rely on conditions where artificial wastewater contained one or a few antibiotics. In the present study, *Chlorella sorokiniana* was used to assess the removal of a mixture of 10 antibiotics selected and tested considering environmentally relevant antibiotic concentrations based on data from the National Health Service (NHS, United Kingdom). The selected antibiotics had a risk quotient > 1 as calculated by the ratio of predicted environmental concentration (PEC) to predicted no effect concentration (PNEC). The experimental antibiotic concentration tested for each antibiotic corresponded to their PEC values. After 19 days of incubation, the β-lactam class (amoxicillin, penicillin V, cephalexin) showed the highest % of removal (51-85), followed by trimethoprim (24), oxytetracycline (6), metronidazole (6), and sulfamethoxazole (2). Different mechanisms, i.e. biodegradation, photodegradation, bioadsorption, and bioaccumulation, were involved at variable range. Increase in algal biomass was observed concomitantly to decrease in the concentration of the tested antibiotics,

suggesting their use as a carbon source for cellular growth. In addition, levels of dissolved NH_4^+, NO_3^-, PO_4^{3-}, and COD (chemical oxygen demand), decreased by 88, 22, 100, and 10 %, respectively. Our study confirmed the ability of *C. sorokiniana* to biodegrade antibiotics while also effectively reducing key nutrient loadings.

Keywords: Microalgae; *Chlorella sorokiniana*; removal; antibiotics; nutrients

INTRODUCTION

Access to clean water is a global priority, as supported by the United Nations Sustainable Development Goal (SDG) 6 that aims to ensure availability and sustainable management of water and sanitation for all. Among the outcome targets to be achieved by 2030 for this SDG, one (6.3) consists in improving the quality of water by reducing pollution, eliminating dumping and minimising release of hazardous chemicals and materials (United Nations, 2015). It also includes halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally. Among the chemical substances polluting waters, active pharmaceutical ingredients have been shown to occur in riverine environments across all continents (Burns et al., 2017; Wilkinson et al., 2022). Antibiotics are chemicals produced by microorganisms (fungi or bacteria) or by chemical synthesis, which have been used for decades in both animals and humans to selectively kill or inhibit the growth of different types of microbes. There are currently over 250 antibiotics that are categorized by their chemical structures or mechanisms of action (Ahmed et al., 2015; Pancu et al., 2021). The negative effects associated with the presence of antibiotics in the environment are of increasing concern as these micropollutants can be detected in effluent of treated wastewaters and

water bodies (Polianciuc et al., 2020). Effluent discharge standards encompass temperature, pH, biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solid (SS), total nitrogen, and total phosphate, but do not include limits for antibiotic concentrations (Kayode et al., 2018; Lu et al., 2016). Presence of antibiotics represents a risk to human health, e.g. by contaminating food, generating allergies, causing chronic toxic impact and digestive tract malfunctions, and selecting for antimicrobial resistance. These antibiotics are not fully metabolised once consumed, and contaminated water needs to be treated to remove them (Polianciuc et al., 2020; Wang et al., 2024).

Methods to treat water can be divided in three categories: physical, chemical, and biological (Ahmed et al., 2021). Physical and chemical treatments are used to treat domestic and industrial wastewater. Biological treatments are based on biological processes or organisms removing contaminants by various mechanisms including bioabsorption, bioaccumulation, biodegradation, and photodegradation. Microorganisms have been particularly well investigated regarding their capacities for wastewater treatment, and among them, microalgae are being used for removal of antibiotics and heavy metals (Abdel-Raouf et al., 2012; Barreto et al., 2019; Hom-Diaz et al., 2022; Nguyen et al., 2021; Ricky et al., 2022; Xiong et al., 2017, 2018). Microalgae can accumulate products of degradation processes into their biomass, which can subsequently be used to produce biofuels, biopolymers, animal feed, supplements, pharmaceuticals, and fertilisers as part of the bioeconomy (Ahmad et al., 2022; Ammar et al., 2022; Halder & Azad, 2019; Koutra et al., 2018; Suleiman et al., 2020).

Many studies on the use of microalgae for removal of antibiotics investigated green microalgae, in particular from the genus *Chlorella*. Hom-Diaz et al. (2022) considered *C.*

sorokiniana (UTEX ID 1663) for the removal of nine antibiotics: azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), ciprofloxacin (CIP), oflaxacin (OFC), norfloxacin (NFC), trimethroprim (TMP), sulfapyridine (SPY) and pipemidic acid (PMA). *C. sorokiniana* showed no ability to remove CLR, and efficiency was variable for other antibiotics, ranging from 73 % (SPY) to 11 % (TMP). In a different study, CIP and amoxicillin (AMX) removal was tested separately in the presence of *C. vulgaris* (Ricky et al., 2022). These authors observed a removal of 37 and 25 % for CIP and AMX respectively. Ndlela et al. (2023) tested co-culture of two *Chlorella* species (*C. vulgaris and C. protothecoides*) in the presence of a mixture of five antibiotics (sulfamethoxazole (SMX), AMX, TMP, OFC, and CLR) added at two different concentrations (10 and 100 ppb). The removal efficiency was 73 % (10 ppb solution) and 47 % (100 ppb solution) for SMX, and 44 % (10 ppb solution) and 55 % (100 ppb solution) for OFC.

In the reports described above, all the antibiotic concentrations were based on empirical criteria and were not calculated considering the risk quotient (RQ). In our study, we aim to test the removal of antibiotics by *C. sorokiniana* under environmentally relevant concentrations calculated based on data obtained from the National Health Service (NHS) in the United Kingdom (UK). For this we evaluated the risk of antibiotic resistance using RQ values based on the ratio of predicted environmental concentration (PEC) to predicted no effect concentration (PNEC) (Supplemental Data, Figure S1). While PEC values can be variable and would be country dependent, well accepted PNEC values for antibiotics are available in the literature (Tell et al., 2019). Previously, Jones et al. (2002) assessed the environmental impact of the 25 most used pharmaceuticals in England in 2000 based on NHS data. The PEC/PNEC ratio exceeded 1.0 for paracetamol, AMX, OTC, and mefenamic acid, indicating potential environmental risk. For our

study, PEC values were calculated using total quantities of antibiotic prescribed in the UK based on the NHS annual prescription report in 2019 (National Health Service, 2020) and considering the % excretion from the drug bank online database (DrugBank online, 2006). Antibiotics with the highest RQ values (AMX, Pen V, MTZ, CEX, CLR, CIP, OTC, TMP, AZM, and SMX) were selected and incubated as a mixture in the presence of *C. sorokiniana* to test the capacity of this microalga to remove them and to determine which process (biodegradation, photodegradation, bioadsorption, and bioaccumulation) was involved in removal. At the same time, we assessed the impact of the antibiotics on the growth of *C. sorokiniana*, as well as the phycoremediation of several nutrients (NH_4^+, NO_3^-, PO_4^{3-}) and changes in the COD.

MATERIALS AND METHODS

Microalgal strain and antibiotics

Chlorella sorokiniana SAG 211-8k (The Culture Collection of Algae at the Goettingen University - Department Experimental Phycology and Culture Collection of Algae (EPSAG)) was obtained from the Mackinder group, Centre for Novel Agricultural Products (CNAP), Department of Biology, University of York.

Antibiotics amoxicillin (AMX), phenoxymethylpenicillin/penicillin V (Pen V), cephalexin (CEX), oxytetracycline (OTC), clarithromycin (CLR), azithromycin (AZM), ciprofloxacin (CIP), sulfamethoxazole (SMX), trimethoprim (TMP), and metronidazole (MTZ) were of analytical grade and purchased from Merck (Gillingham, UK). They were dissolved in methanol to prepare individual stock solutions at 1 mg/ml and stored at -20 ˚C before use.

Growth conditions and experimental set-up

C. sorokiniana was maintained in BG11 prepared according the recipe provided by the UTEX culture collection of algae (Supplemental Data, Table S1). Solid medium was prepared by adding 15 g/L of agar, and autoclaved before adding sterile sodium thiosulfate pentahydrate at 1 mM final concentration.

Algal liquid cultivation was carried out in 50 ml of sterile BG11 broth medium contained in 250 ml flasks, under fluorescent light at 40 µmol m^{-2} s^{-1} (Skye Instruments, UK), a 16/8 hour light/dark cycle, shaking at 130 rpm, and at 25 ˚C. Flasks were wrapped with aluminum foil to test darkness condition.

Four different experimental conditions (Table 1) were tested in triplicate. Treatment A (CS-BG11-ABS; *C. sorokiniana* grown in BG11 medium containing antibiotics), C (BG11-ABS-light; BG11 medium containing antibiotics and incubated under light condition), and D (BG11-ABS-dark; BG11 medium containing antibiotics and incubated under dark condition) were spiked with a mixture of ten antibiotics added at different concentrations corresponding to ten times their PEC values (µg/ml) calculated as described in the section below (Table 2). The control (treatment B, CS-BG11-MeOH; *C. sorokiniana* grown in BG11 medium containing methanol (0.5% v/v) and no antibiotics) was run under the same conditions as treatments A, C, and D, but methanol was added instead of the mixture of antibiotics. Treatments A, B, and C were conducted under the same 16/8 hour light/dark cycle. Cultures were started at 1 X 10^5 cells/ml for treatment A and B. Growth was evaluated daily using a Countess II FL automated cell counter (Invitrogen, USA) and by measuring change in absorbance at 750 nm using a Cary 50 Bio UV-visible spectrophotometer (Varian, USA). For treatments A, B, C, and D samples were collected on day 0, 2, 6, 9, 14, and 19. While treatments C and D provided only liquid samples,

samples from treatments A and B were centrifuged at 4,000 rpm for 20 min to separate the microalgal cells from the liquid medium; the supernatant was collected, and the algal cells were shock-frozen in liquid nitrogen and then stored at -20 ˚C. Liquid fractions were filtered with 0.20 µm non-pyrogenic/endotoxin-free syringe filters (SARSTEDT AG & Co. KG, Nümbrecht, Germany) before determination of antibiotics concentration and nutrient content.

Predicted environmental concentration (PEC) and risk quotient (RQ) calculations

The calculation of PEC (concentration in raw wastewater) for 82 antibiotics and antimicrobial compounds was based on equation 1.

$$PEC = \frac{\text{(Total mass precribed * Excretion rate)}}{\text{Total amount of wastewater generated}} \quad (1)$$

For this equation, the total mass prescribed (mg) for each compound was taken from the annual prescription report of the NHS database (National Health Service, 2020) (Supplemental Data, Table S2). Excretion rate corresponded to the % of the pharmaceutical excreted unchanged by patients (DrugBank online, 2006). The total amount of wastewater generated was calculated using equation 2, where P is the number of inhabitants in UK in 2019 (66,800,000) (UK Office for National Statistics, 2021), V is the volume of waste water per day per capita (200 L/day/person) (Bound & Voulvoulis, 2006; Burns et al., 2017; Sabaliunas et al., 2003), and 365 the number of days in one year.

$$\text{The total amount of wastewater generated} = P * V * 365 \text{ days} \quad (2)$$

Risk quotient (RQ) was calculated according to equation 3, for which PNEC values were extracted from Tell et al. (2019).

$$RQ = \frac{PEC}{PNEC} \quad (3)$$

Determination of antibiotic concentration

Concentration of the ten selected antibiotics was measured in filtered supernatants using an Agilent Infinity II 1260 HPLC with MSDiQ single quadrupole mass spectrometer (Agilent Technologies, United States). Instrument control, data acquisition and processing were performed by Agilent OpenLab CDS v2.6. Solvent A was LC-MS grade water containing formic acid (0.1 %, v/v) and solvent B was LC-MS grade methanol containing formic acid (0.1 %, v/v). A gradient method was employed at a flow rate of 0.45 ml/min: initial 80 % A - 20 % B to 0 % A - 100 % B at 9 min where it was held until 15 min before returning to the initial composition 80 % A - 20 % B and flushing 6 min before injection of the next sample. The oven containing a Zorbax SB-C18 column (4.6 x 100 mm, 3.5 µm) was set at 40 ˚C, and the injection volume was 10 µl. The eluant from the first 3 min of the run was diverted to waste to avoid involatile salts fouling the detector. The detector was equipped with an electrospray ionisation source operated in positive ion mode. The following ions were monitored, corresponding to the protonated molecules of the target analyses: m/z 366.1 for AMX, 749.5 for AZM, 348.3 for CEX, 332.2 for CIP, 748.6 for CLR, 172.3 for MTZ, 461.2 for OTC, 351.1 for Pen V, 254.1 for SMX and 291.2 for TMP. For quantification, individual antibiotic solutions (1,000 mg/L) were prepared, and then the antibiotics were mixed to prepare a solution at 1,000 µg/L for each of them. To obtain a standard curve (Supplemental Data, Figure S2), serial dilutions to 500, 100, 50, 5, 1,

and 0 µg/L were prepared using deionised water/methanol (80:20, v/v) in 50 ml volumetric flasks. These solutions were then transferred into amber glass tubes and stored at -20 ˚C.

Determination of antibiotic removal

The percentage of total antibiotic removal was calculated as previously described (Ricky et al., 2022; Xiong et al., 2017) using equation 4.

$$\text{Total removal (\%)} = \frac{\text{Initial antibiotic concentration (Ci) - Residual concentration (Cr)}}{\text{Initial antibiotic concentration (Ci)}} \text{ X } 100 \qquad (4)$$

Different mechanisms are involved in removal of antibiotics. Treatment A was used to estimate the contribution of bioadsorption (Rad) to this process. For this, cell pellets were redissolved with 1 ml of deionized water and centrifuged at 5,000 rpm for 10 min, as previously described (Xiong et al., 2016 and 2017). The supernatants were filtered and stored at -20 ˚C before antibiotic analysis. Bioaccumulation of antibiotics (Rac) inside the algal cells was determined following the procedure reported by Xiong et al. (2016, 2017): 1 ml of extraction solution (dichloromethane:methanol; 1:2 v/v) was added to the cell pellet, which was then vortexed for 1 min, and then sonicated for 1 hour. The supernatant was recovered by centrifugation at 15,000 rpm for 10 min and filtered 0.2 µm. It was then dried by evaporation at room temperature, redissolved in a mixture of deionized water:methanol (1:1 v/v), filtered again, and stored at -20 ˚C before analysis by HPLC-MS.

Photodegradation was determined by calculating the difference in antibiotic concentration between incubation under light and dark conditions in the absence of algal cells (treatments C and D). Percentage of abiotic condition removal (Ra) was quantified by equation 5 (Hom-Diaz et al., 2022; Ricky et al., 2022).

$$Ra\ (\%) = \frac{\text{Residual concentration (abiotic dark) - Residual concentration (abiotic light)}}{\text{Initial antibiotic concentration (Ci)}} \text{ X } 100 \quad (5)$$

Biodegradation of antibiotics can occur through several processes in living organisms, and the contribution of *C. sorokiniana* was evaluated using equation (6) (Ricky et al., 2022; Xiong et al., 2016; Xiong et al., 2017):

$$\text{Biodegradation } (\%) = \frac{Ci - Cr - Rad - Rac - Ra}{Ci} \text{ X } 100 \quad (6)$$

In this equation, Ci corresponds to the initial antibiotic concentration, Cr to the residual antibiotic concentration, Rad to the antibiotic bioadsorption concentration, Rac to the antibiotic bioaccumulation concentration, and Ra to the abiotic condition concentration by photodegradation.

Determination of nutrient removal

At least 3 ml per sample (treatment A and B; days 0, 2, 6, 9, 14, 19) were filtered through 0.20 µm filter disks membranes (SARSTEDT AG & Co. KG, Nümbrecht, Germany). Nutrient concentrations were determined using the Seal Analytical AutoAnalyzer 3 (AA3 HR., Seal Analytical Inc., UK). AutoAnalyser control and evaluation used AACE7 Seal Analytical software version 7.10 SP3 2018 (AA3 HR., Seal Analytical Inc., UK). Standard methods provided by Seal Analytical were considered for quantification: Nitrate (NO_3^-) G-109-94 Rev.1 (Multitest MT7/MT8), Ammonium (NH_4^+) G-102-93 Rev.1 (Multitest MT7/MT8), and Phosphate (PO_4^{3-}) G-103-93 Rev. 10 (Multitest MT7/MT8). Standard curves for each nutrient (NO_3^-, NH_4^+, and PO_4^{3-}) (Supplemental Data, Figure S3) were obtained by preparing a stock solution of 1,000 mg/L, and then serial dilutions to 2.0, 1.5, 1.0, 0.5, and 0 mg/L with deionised water. Dilutions were stored at 4 ˚C before analysis by AutoAnalyser. Four reagents (ammonium molybdate

reagent, ascorbic acid reagent, sulfuric acid reagent, and system wash solution; Supplemental Data, Table S3) were used as mobile phase. The parameters for the nutrient analysis method were as follows: Water check; Bubble pattern (liquid (ms) min 500 max 2000, Variation max 2%); Light energy (Reference (mV) min 250 max 2500, Sample ref. ratio min 0.3; Water baseline check (Drift (mAU/hr) max 5, Noise (mAU) max 0.5. Reagent check; Reagent baseline check (Absorbance (AU) min 0 max 0.1, Drift (mAU/hr) max 6, Noise (mAU) max 0.5). Run check; Light energy (Reference (mV) min 250 max 2500, Baseline drift and Sensitivity drift (%) max 5, Sensitivity min 0.1, Carryover (%) max 2, Calibration Coeff. min 0.9); Temperature; Std. Heating bath 3 and 4 at 40 ˚C, Internal distill bath 1 and 2 at 40 ˚C, External ADM 25 ˚C. Total run time was 1 min per sample, followed by 12 sec per sample to wash. Absorbances were measured at 520 nm for nitrate, 660 nm for ammonia (salicylate), and 660 nm for phosphate.

Percentage of total nutrient removal for each nutrient was calculated using equations (7), (8), and (9):

$$NH_4^+ \text{ removal (\%)} = \frac{\text{Initial concentration (Ci) - Residual concentration (Cr)}}{\text{Initial concentration (Ci)}} \text{ X } 100 \quad (7)$$

$$NO_3^- \text{ removal (\%)} = \frac{\text{Initial concentration (Ci) - Residual concentration (Cr)}}{\text{Initial concentration (Ci)}} \text{ X } 100 \quad (8)$$

$$PO_4^{3-} \text{ removal (\%)} = \frac{\text{Initial concentration (Ci) - Residual concentration (Cr)}}{\text{Initial concentration (Ci)}} \text{ X } 100 \quad (9)$$

Determination of chemical oxygen demand (COD)

Two ml of supernatant from treatment A and B were used with the LCK 514 Chemical Oxygen Demand kit (100 - 2000 mg/L O_2, Hach, Colorado, United States). The samples were incubated for 15 min at 170 ˚C in a High Temperature Thermostat HT200S (Hach, Colorado,

United States). Chemical oxygen demand values were read using a DR 1900 instrument (Hach, Colorado, United States), and COD removal calculated according to equation 10.

$$\text{COD removal (\%)} = \frac{\text{Initial concentration (Ci)} - \text{Residual concentration (Cr)}}{\text{Initial concentration (Ci)}} \text{ X } 100 \qquad (10)$$

Algal cell dry weight (CDW) quantification

Frozen algal cell pellets were dried at -108 ˚C under a pressure of 0.6 mbar using a Heto powerdry ll 1500 freeze dryer (Thermo Scientific, USA). The dried microalgal biomass was weighed and stored at room temperature.

Elemental analysis (C, N, and C/N)

Carbon and nitrogen content, and C/N ratio of *C. sorokiniana* biomass were determined using a Thermo Finnigan Flash EA 1112 elemental analyser (ThermoFisher Scientific, Waltham, MA, U.S.A.). Instrument control and data acquisition used the Eager smart software v.1 2016 (ThermoFisher Scientific, Waltham, MA, U.S.A.). Aspartic acid (certified number 363797–25-Nov-25, Elemental Microanalysis, Devon, UK) was used as a standard for calibration and drift correction. Approximately 1-3 mg of algal cell samples or standard were placed in an 8 × 5 mm tin foil capsule (Elemental Microanalysis, Devon, UK) and weighed before combustion. The elemental analysis method parameters were as follows: the combustion reactor containing copper oxide and silvered cobaltous oxide was held at 900 ˚C; the reduction reactor containing reduced copper was held at 840 ˚C; helium carrier gas flows were 140 ml/min and 100 ml/min; and sample introduction coincided with a pulse of oxygen for 10 sec at 250 ml/min. Combustion gases were detected with a thermal conductivity detector. Total run time was 5 min per sample.

Statistical Analysis

Results were expressed as the average of three independent experiments ± standard deviation (SD). Potential significant differences in algal cell density, algal cell dry weight, algal elemental content (C, N, C/N), and nutrient removal (nitrate, ammonium, phosphate, COD) between treatments with and without antibiotics on the last day of the experiment (day 19) were assessed. Given the limited sample size (3 values per treatment and condition), the Mann-Whitney test (level of significance fixed at p-value ≤ 0.05) was considered and calculations made with GraphPad Prism version 10.3.0 for Windows (2024).

Results

Determination of predicted environmental concentrations (PEC) and risk quotients (RQ) for selected antibiotics

Based on analysis of UK NHS prescription data, calculated PEC values for 82 antibiotics ranged between 2.62 x 10^{-5} to 3138.74 µg/L. These values were used subsequently to estimate the risk quotients, which ranged between 12,555 and to 5 x 10^{-5} (Supplemental Data, Table S2). The antibiotics with the top ten RQ and PEC values (Table 2) were considered further to prepare the mixture of antibiotics to be tested. Their final concentrations ranged from 0.02 (SMX) to 3.14 mg/L (AMX).

Influence of mixed antibiotics on microalgal growth

The growth of *C. sorokiniana* SAG 211-8k in presence (treatment A) and absence (treatment B) of mixed antibiotics was monitored daily for 19 days. As observed in Figure 1, the growth curve obtained for the two treatments showed similar trends. The exponential

phase of growth was reached after 3 days and last until day 6, from which the growth slightly slowed down. Cell number continued to increase in both conditions, and the highest level in presence of mixed antibiotics was observed from day 9. Cells entered exponential phase from days 10 to 15, and then a short phase of algal growth was observed. At the end of the incubation time, the cell number was higher in presence of mixed antibiotics, with a final cell density of $2.14 \times 10^7 \pm 0.01$ compared with $1.81 \times 10^7 \pm 0.46$ cells/ml, respectively (Figure 1 and Supplemental Data, Table S4); this difference was not statistically significant. These growth curves and final cell densities were very similar to what was observed in a separate experiment where *C. sorokiniana* was incubated in absence of methanol and of mixed antibiotics (Supplemental Data, Figure S4).

Algal cell dry weight (CDW)

The dry weight of *C. sorokiniana* cells was determined for treatment A (CS-BG11-ABS) and B (CS-BG11-MeOH) over 19 days. The quantity of biomass between treatment A and treatment B was similar during the first 7 days. Treatment B showed higher cell biomass than treatment A on day 9. On day 14, the biomass in treatment A was higher than in treatment B, and that was maintained until the end of the experiment (Figure 2 and Supplemental Data, Table S4). However, these differences were not statistically significant.

Antibiotics removal and mechanisms involved

Percentages of individual antibiotic removal ranged between 7.5 (AZM) and 100 % (AMX) (Figure 3). Based on these values, antibiotics were split in 3 groups: high, intermediate, and low removal.

Amoxicillin, OTC, CEX and Pen V were completely or almost completely eliminated under the conditions tested. Removal of AMX, OTC, and Pen V followed the same trend throughout the experimental period, with most of it occurring between day 2 and 6. After day 6, AMX and OTC were completely removed, while a limited amount of Pen V remained in the culture medium until the end of the experiment. Total removal of CEX was completed at a more linear rate between day 2 and 14.

Metronidazole and TMP represented the intermediate removal group. Metronidazole showed a gradual removal throughout the experimental period, with 31.6 % removal at day 19. Trimethoprim exhibited a rapid increase in removal from day 0 to day 2, and then showed a trend similar to MTZ up to the end of the experiment at which 53.8 % were removed.

The low antibiotic removal group included antibiotics showing less than 15 % of removal: AZM, 7.5 %; CLR, 8.3 %; SMX, 10.5 %; CIP, 12.1 %. Within this group, the fate of AZM, CLR, and CIP was similar, with very limited removal between day 0 and 9, and then a slight linear increase until day 19. Conversely, most of the SMX was removed during the first half of the experiment.

Different mechanisms, namely photodegradation, bioadsorption, bioaccumulation, and biodegradation were investigated for the removal of the ten selected antibiotics. Results presented in Figure 4 and Supplemental Data, Table S5 showed that these mechanisms had different contributions to antibiotic removal. The primary removal mechanism for most of them was biodegradation. This is particularly true for AMX, Pen V, CEX, AZM, MTZ, SMZ, and TMP. The second most important mechanism was photodegradation (Supplemental Data, Table S6). Interestingly, it was the main mechanism of removal for OTC, CLR, and CIP.

Bioadsorption and bioaccumulation were also observed for some antibiotics, but played a minor role, as the values were below 1 % for each of the antibiotics tested.

Removal of nutrients

Results obtained for the different parameters tested (Figure 5) were very similar between the two physiological conditions tested as no significant variations were observed.

The variation in organic carbon demand was estimated by calculating changes in the chemical oxygen demand (COD) (Figure 5A). Starting from an initial value of 2,476 mg/L (treatment A) and 2,510 mg/L (treatment B) of O_2, final values for treatment A (CS-BG11-ABS) and treatment B (CS-BG11-MeOH) were 2,275 and 2,277 mg/L, respectively. This indicated a 10 % and 8 % decrease of COD under the conditions tested, and this process was mostly linear during the experiment.

The initial nitrate concentration in the medium was 277 mg/L (treatment A) and 278 mg/L (treatment B), and reduced to 216 mg/L in treatment A (22 % removal) and to 224 mg/L in treatment B (19 % removal) (Figure 5B). Interestingly, the consumption of nitrate was not linear during the growth of *C. sorokiniana*, as very limited amounts were utilized during the first half of the experiment, and most was used after 9 days of incubation. This is in contrast with changes in the content of ammonium (Figure 5C; initial concentration of 0.22 mg/L (treatment A) and 0.25 mg/L (treatment B)). Indeed, 95 % of the ammonium was used after 5 days under both treatments, and a slight release of this compound was monitored by day 19.

Finally, Figure 5D showed that *C. sorokiniana* was very efficient at using phosphate (initial concentration of 6.1 mg/L (treatment A and B)). Although its quantity varied only slightly

during the first two days of incubation, it was completely depleted in the medium by the end of the experiment.

Elemental analysis (C, N, and C/N) of algal cells

Carbon (C) and nitrogen (N) content, as well as carbon/nitrogen ratio (C/N), were determined in presence and absence of antibiotics. The most abundant element was C, and its percentage increased in both treatments through the experiment. It can be seen that treatment A (CS-BG11-ABS) had slightly higher carbon content than treatment B (CS-BG11-MeOH) on day 19 with values of 50.86 % and 49 %, respectively (Figure 6). There was no significant difference between treatments A and B.

The evolution of N percentage followed the same pattern in treatments A and B, with gradual decrease from the start to the end of the experiment. On day 19, the % N in treatments A and B was 8.31 and 7.39 %, respectively (Figure 6), but this difference was not supported statistically.

The C/N ratio in treatments A and B followed the same trend as the C percentage. It increased during the experiment, and the highest values for both treatments were observed on day 19 at 6.12 and 6.64 for treatments A and B, respectively (Figure 6). These values were not statistically different.

DISCUSSION

Influence of antibiotics on algal growth

As incubation of *C. sorokiniana* in absence of methanol and of mixed antibiotics was conducted in a separate experiment, it is not possible to rule out that the presence of

methanol may negatively impact the growth of *C. sorokiniana* and its capacities to remove nutrients and antibiotics. In this context, we observed a cell density slightly higher in presence of mixed antibiotics dissolved in methanol than in presence of methanol only. Similar results were described for *Chlorella* species in previous reports. Ricky et al. (2022) tested *C. vulgaris* BDU GD003 grown in BG11 medium containing five mg/L of AMX or CIP for seven days. At the end of the experiment, cell densities were 6.75 x 10^6 and 4.85 x 10^6 cells/ml when comparing AMX treatment and control condition, respectively, and 7.01 x 10^6 and 4.71 x 10^6 cells/ml for CIP treatment and control condition, respectively. In line with this, Yazdi et al. (2018) investigated the removal by *C. vulgaris* of Pen antibiotic added at 2 mg/L in BBM medium for eight days. They observed a higher cell concentration (4.01 x 10^7 cells/ml) in the presence of the antibiotic compared to in the absence of Pen (0.53 x 10^7 cells/ml).

Frascaroli et al. (2024) used three green microalgae, *Chlamydomonas acidophila* CCAP11/133, *Auxenochlorella protothecoides* CCAP 211/7A (formerly known as *Chlorella protothecoides*) and *Tetradesmus obliquus* CCAP 276/6B, to assess the removal of an antibiotic mixture comprising CIP, CLA, OFL, TMP, ERY, MTZ, and SMX (100 µg/L final concentration for each antibiotic) in BG11 medium. Different influence of the antibiotics was observed on these algae. Slightly higher cell density was observed for *A. protothecoides* in their presence (2.7 x 10^6 cells/ml compared to 2 x 10^6 cells/ml), while an opposite trend was monitored for *C. acidophila* (6.5 x 10^6 and 7.5 x 10^6 cells/ml) and *T. obliquus* showed (4.7 x 10^6 and 6 x 10^6 cells/ml).

Antibiotic removals by green microalgae

Hom-Diaz et al. (2022) reported the cultivation of *C. sorokiniana* UTEX 1663 for 14 days in a TAP medium containing a mixture of ten pharmaceutical compounds (9 antibiotics: AZM, CLR, CIP, TMP, ERY, OFC, NFC, SPY, PMA; one antidepressant: VFX) added at a final concentration of 100 µg/L each. They assessed the % removal of each of these compounds, and the mechanisms involved photodegradation, biodegradation, and sorption). Their results were as follows: AZM (58, 1, 9, and 12 %, respectively), CLR (0, 0, 0, 0 %, respectively), CIP (51, 49, 0, 0 %, respectively) and TMP (11, 2, 0, 1 %, respectively). Kiki et al. (2020) tested the potential of antibiotic removal by *C. vulgaris* FACHB-24 in synthetic wastewater containing a mixture of ten antibiotics (AZM, CLR, TMP, SMX, SMR, SMM, ROX, LOM, LEV, and FLU) at 100 µg/L for 40 days. The values of % total removal, % bioadsorption, and % bioaccumulation observed were 79, 2, 4 %, for AZM and 81, 3, 5 % for CLR; they were 31, 2, 0 % for TMP, and 75, 1, 0 % for SMX. When comparing these published data with our results, AZM removal was less efficient in our experimental set-up with 7.5 % total removal, and less than 1 % of photodegradation, bioadsorption, bioaccumulation, and biodegradation. We observed limited CLR removal (8.3 %) compared to Kiki et al. (2022), but more than reported by Hom-Diaz et al. (2022). In contrast, we observed a more efficient removal of TMP (53.8 %) compared to these two studies. For CIP, our obtained values of % total removal (12.1) and % photodegradation (6.6), with nearly 0 % (biodegradation and bioadsorbtion), were in the same range as Hom-Diaz et al. (2022). For SMX, we observed lower % total removal (10.5) compared to Kiki et al. (2020), but similar values of bioadsorbtion and bioaccumulation. The more efficient % total removal observed by these authors may be related to the longer experimental time, i.e. 40 days compared to 19.

Zhou et al. (2014) collected raw wastewater (influent) containing 50 organic compounds and used *C. pyrenoidosa* (Cp) FACHB-9 and *C. vulgaris* (Cv) FACHB-31 to treat it for seven days. CIP (29.3 ng/L), CLR (17.3 ng/L), SMZ 50.7 (ng/L), and TMP (27 ng/L) were among the compounds contained in this wastewater, and their % of removal were as follow: CIP: 74.4 % (Cp) and 100 % (Cv); CLR: 100 % by both Cp and Cv; SMX: no removal by either Cp or Cv; TMP: 28.1 % (Cp), 0 % (Cv). Our research showed lower efficiency in the removal of CIP (12.1 %), CLR (8.3 %) and SMX (10.5 %), but higher efficiency for TMP (53.8 %).

de Wilt et al. (2016) conducted experiments with *C. sorokiniana* CCAP211/8K grown in urine, anaerobically treated black water (AnBW) and synthetic urine to remove six pharmaceutical compounds (diclofenac, ibuprofen, paracetamol, metoprolol, carbamazepine and TMP) spiked at 100 - 350 µg/L for 31 days. They observed limited removal of TMP in urine and synthetic urine (60 %) and in AnBW (40 %), possibly due to high resistance of TMP to degradation under aerobic conditions. Photodegradation and sorption accounted for 20 % of removal each, and were the main removal mechanisms involved, in contrast with what was observed in our study.

Chlorella species (*C. vulgaris* and *C. pyrenoidosa*) were also used to treat growth medium (BG11 and BBM) containing a single antibiotic. Ricky et al. (2022) cultured *C. vulgaris* BDU GD003 in BG11 spiked with CIP or AMX at 5 mg/L for seven days. Percentage of total removal and contribution of different mechanisms (photodegradation, bioadsorption, bioaccumulation, and biodegradation) were: 36.9, 0.2, 5.7, 2.9, and 28.1 % for CIP; 24.7, 6, 4.6, 2.7, 11.5 % for AMX. In our experiments, CIP showed higher % of photodegradation and lower % of total removal and contribution of other mechanisms. For AMX, we quantified higher % of

total removal and biodegradation, similar % photodegradation, and lower % of bioadsorption and bioaccumulation. Yazdi et al. (2018) treated BBM medium containing Pen V at 2 mg/L with *C. vulgaris* for eight days, and obtained % total removal of 92.75. We observed similar value in our experimental setting with *C. sorokiniana* of Pen V (85.8 %).

Li et al. (2015) grew *C. pyrenoidosa* FACHB-1220 in BG11 medium spiked with AMX or cefradine at 100, 200, 300, 400, and 500 mg/L for 48 hours. Value of % total removal were 97.36, 95.12, 94.69, <90, and <90 % for AMX, and 22.52, 11.13, 7.28, 8.47, and 7.43 % for cefradine under the different conditions tested. Cefradine is part of the cephalosporin/β-lactam class of antibiotics, like CEX (DrugBank online, 2006) considered in our study. We observed 100 % total removal for CEX, suggesting that *C. sorokiniana* SAG211-8K was more efficient than *C. pyrenoidosa* to remediate this antibiotic. Both species of *Chlorella* were very efficient at removing AMX. Xiong et al. (2017) studied the removal of LEV included in BBM medium at 1 mg/L by *C. vulgaris*, and observed 16 % of total removal. Levofloxacin is an antibiotic of the class fluoroquinolone, like CIP (DrugBank online, 2006). In our experiment, we showed value for the removal of this antibiotic (12.1 %).

Nutrient removals

We observed a COD removal of 8 % and 10 % under control conditions and in presence of antibiotics respectively. These values were lower than those measured in other studies investigating phycoremediation of polluted waters by *Chlorella* species. Ji et al. (2018) cultivated *C. vulgaris* in synthetic wastewater and measured 60 % of COD removal. Chen et al. (2017) determined that 60 % of the COD was removed from artificial wastewater by *C. sorokiniana*. Mujtaba et al. (2017) reported an experiment during which 72 % of the COD was

removed from synthetic wastewater by using the microalga *C. vulgaris.* Tran et al. (2021) used *C. variabilis* TH03 for treating domestic wastewater, and COD showed a 99.9 % removal. Gowd et al. (2024) reported the % COD removal from university, municipal, and dairy wastewater using *Chlorella sp.* being of 76, 94, and 93 %, respectively. Hasan et al. (2014) treated swine wastewater by *C. vulgaris* (UTEX 2714) and showed COD removal at 58.9 %. Asadi et al. (2019) investigated the % of COD removal by two *Chlorella* species (*C. vulgaris* and *C. sorokiniana* pa.91) grown in dairy wastewater effluents, and both *Chlorella* species allow to reach a 99.9 % COD removal in both preliminary and secondary effluents. Zhang et al. (2019) treated unsterilized piggery wastewater using *C. pyrenoidasa*, and COD removal was 20 - 55.1 % depending on experiment conditions. Ji et al. (2021) used *C. vulgaris* FACHB-8 to treat stimulated domestic sewage, obtaining 34.8 % of COD removal. Tao et al. (2017) used *C. vulgaris* (SAG211-11b) for treating two wastewaters (anaerobic digestion of pulp and paper industry, and municipal wastewaters) and determined values of 27.6 and 55.4 %, respectively. When considering values of % COD removal, swine and piggery wastewater generally showed lower values than other types of wastewaters.

Nitrate removal was slightly over 20 % in our experiments, without significant differences in presence or absence of the antibiotic mixture. These values were lower than previously reported in several studies. It can be suggested that the combination of antibiotics used may have inhibited nitrogen metabolism (DrugBank online, 2006), while slightly stimulating algal growth. When considering the research of Kiki et al. (2020) in which *C. vulgaris* FACHB-24 was used for treating synthetic wastewater as described in previous section, lower % of nitrate removal (97, 96, and 90 %) where observed in the presence of increasing quantities of

antibiotics. Daneshvar et al. (2018) grew *C. vulgaris* in BBM medium for eight days and determined the % of nitrate removal to be 70.48. Gowd et al. (2024) reported % of nitrate removal higher than 90 % in university, municipal, and dairy wastewater by *Chlorella* sp. Sayadi et al. (2016) treated municipal wastewater by *C. vulgaris*, and showed a % of nitrate removal of 89.8.

In our experiments, we obtained an ammonium removal of 88 %. This value is higher than those reported in previous studies. Ji et al. (2021) measured 39.8 % of ammonium removal in stimulated domestic sewage treated by *C. vulgaris* FACHB-8. Tao et al. (2017) reported % ammonium removal by *C. vulgaris* SAG211-11b after treatment of anaerobic digestion of pulp and paper industry (44 %), and municipal wastewater (23.8 %). Chen et al. (2017) reported ammonium removal of 60 % after treatment of artificial wastewater by *C. sorokiniana*. Mujtaba et al. (2017) obtained 70 % of ammonium removal in synthetic wastewater by *C. vulgaris*. Hasan et al. (2014) measured removal of 92.5 % of ammonium in swine wastewater treated with *C. vulgaris* (UTEX 2714). Tran et al. (2021) used *C. variabilis* TH03 to test remediation of domestic wastewater and showed 97.7 % of ammonium removal. Finally, Refaay et al. (2022) used *C. sorokiniana* to treat industrial wastewater and showed 87.8 % of ammonium removal, a value quite close to what we observed in our study.

Phosphate removal was complete in our experimental settings. Most of the studies conducted so far showed also efficient removal of phosphate when using *Chlorella* species. Mujtaba et al. (2017) determined that 66 % of phosphate was removed when using *C. vulgaris* to treat synthetic wastewater. Tao et al. (2017) used *C. vulgaris* SAG211-11b to treat anaerobic digestion of pulp and paper industry and municipal wastewater. They measured phosphate

removal % of 98.9 and 99 %, respectively. Tran et al. (2021) showed 99.9 % of phosphate removal in domestic wastewater treated by *C. variabilis* TH03. Sayadi et al. (2016) used *C. vulgaris* to treat municipal wastewater and showed phosphate removal % of 88. Gowd et al. (2024) reported 94, 85, and 85 % of phosphate removal in municipal, dairy, and university wastewaters when considering *Chlorella sp.* Refaay et al. (2022) treated industrial wastewater with *C. sorokiniana* and showed 100 % of phosphate removal. Additionally, Kiki et al. (2020) observed % of phosphate removal increasing from 77 to 96 % in line with increased antibiotic concentration in synthetic wastewater treated with *C. vulgaris* FACHB-24.

Biomass and elemental analysis of algal cells

We observed an algal biomass of 0.75 g/L for *C. sorokiniana* SAG 211-8k in presence of the mixture of antibiotics in BG11. This value is slightly higher than in the study by Montoya-Vallejo et al. (2023) (0.705 g/L) who grew *C. sorokiniana* in the Chu13 medium. These data are in line with those obtained for other *Chlorella* species, i.e. *C. vulgaris* TISTR 8261 in Chu 13 medium at 0.75 g/L (Kitcha & Cheirsilp, 2014), and *C. vulgaris* grown in BBM media spiked with 0.4 mg/L of penicillin (0.67 g/L; Yazdi et al. (2018)).

Moreover, microalgal biomass depends on the types of wastewaters considered for phycoremediation. Chen et al. (2018) cultivated *C. sorokiniana* and *C. vulgaris* ESP-6 in anaerobically digested wastewater and obtained biomass values of 0.54 and 0.45 g/L, respectively. Asadi et al. (2019) investigated *C. sorokiniana* pa.91 and *C. vulgaris* grown in dairy wastewater effluents from treatment plants (preliminary and secondary treated effluents). The biomass of *C. sorokiniana* pa.91 was 1.766 and 1.054 g/L, and values of 1.843 and 1.125 g/L were determined for *C. vulgaris*. Tran et al. (2021) used *C. variabilis* TH03 to treat domestic

wastewater, and observed values ranging between 1.52 and 1.72 g/L. Zhang et al. (2019) estimated *C. pyrenoidosa* biomass after treating it with unsterilized piggery wastewater, which showed 0.88 - 1.31 g/L after treatment of unsterilized piggery wastewater.

The C and N contents of *C. sorokiniana* SAG211-8k were 50.86 and 8.31 respectively after being incubation in presence of the ten antibiotic solutions. These values are higher than most of those obtained for *Chlorella* biomass presented in Table 3.

CONCLUSIONS

We showed that *C. sorokiniana* has potential for phycoremediation of antibiotics, in particular AMX, PenV, CEX, and TMP when mixed with other antibiotics and under laboratory conditions. We also observed that the presence and removal of the antibiotics did not alter the growth of the microalgae. In the future, it would be interesting to analyse the degradation products of some of these antibiotics to learn more about the mechanisms involved, as well as to investigate how new microalgal (*C. sorokiniana*) – bacterial consortia contribute to antibiotics and nutrients removal.

TABLE 1: Experimental conditions tested in this study. Treatments A, B, and C were conducted under a 16/8 hour light/dark cycle.

Treatment	Experimental Condition	*C. sorokiniana*	Antibiotics	Illumination
A (CS-BG11-ABS)	Antibiotic test	+	+	+
B (CS-BG11-MeOH)	Control	+	-	+
C (ABS-BG11-light)	Abiotic light	-	+	+
D (ABS-BG11-dark)	Abiotic dark	-	+	-

TABLE 2: Top ten antibiotics PEC and RQ values.

Name	PEC (µg/L)	Risk Quotient
Amoxicillin (AMX)	3140	12555
Penicillin V (Pen V)	620	10387
Metronidazole (MTZ)	180	1364
Cephalexin (CEX)	150	1897
Clarithromycin (CLR)	110	1386
Ciprofloxacin (CIP)	80	1365
Oxytetracycline (OTC)	80	153
Trimethoprim (TMP)	80	153
Azithromycin (AZM)	30	1264
Sulfamethoxazole (SMX)	20	26

TABLE 3: Elemental analysis of microalgal biomass.

Microalgae	C (%)	N (%)	Condition	References
C. sorokiniana SAG211-8k*	50.86	8.31	BG11 with 10 antibiotics	This study
C. sorokiniana SAG211-8k*	49	7.39	BG11 with MeOH	This study
Chlorella sp.	44.33	8.53	NPK fertilizer (N 24%, P 24%, K18%) 0.5 g/l	Rendón-Castrillón et al. (2021)
C. vulgaris	45.8	4.6	Open pond with CO_2 supplier	Xu et al. (2011)
C. vulgaris	42.51	6.64	BBM medium with CO_2	Wang et al. (2013)
C. vulgaris (CCAP 211/11B)	43.9	6.7	TAP medium	Kebelmann et al. (2013)
C. vulgaris (UTEX 2714)	45.9	11.6	Cultivation in swine WW	Hasan et al. (2014)
C. vulgaris	44.5	9.6	TAP medium	Thangalazhy-Gopakumar et al. (2012)

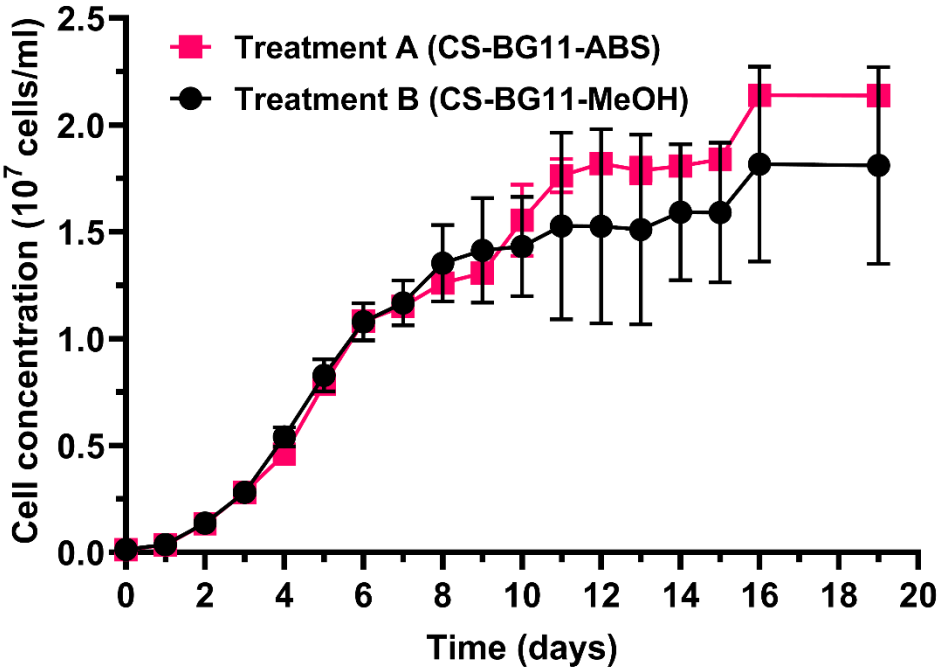

FIGURE 1: Growth of *C. sorokiniana* SAG 211-8k in presence and in absence of mixed antibiotics.

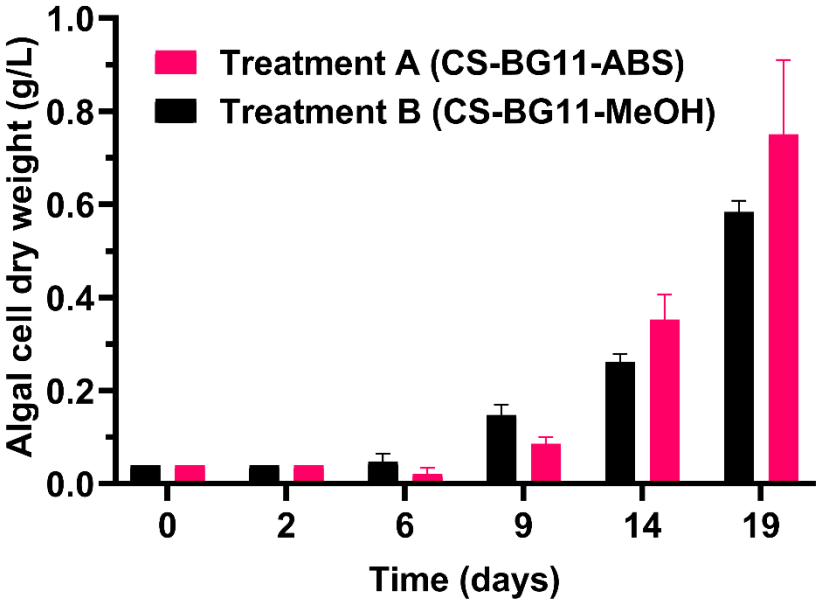

FIGURE 2: Changes in algal cell dry weight (g/L) measured in treatment A (CS-BG11-ABS) and B (CS-BG11-MeOH) over 19 days.

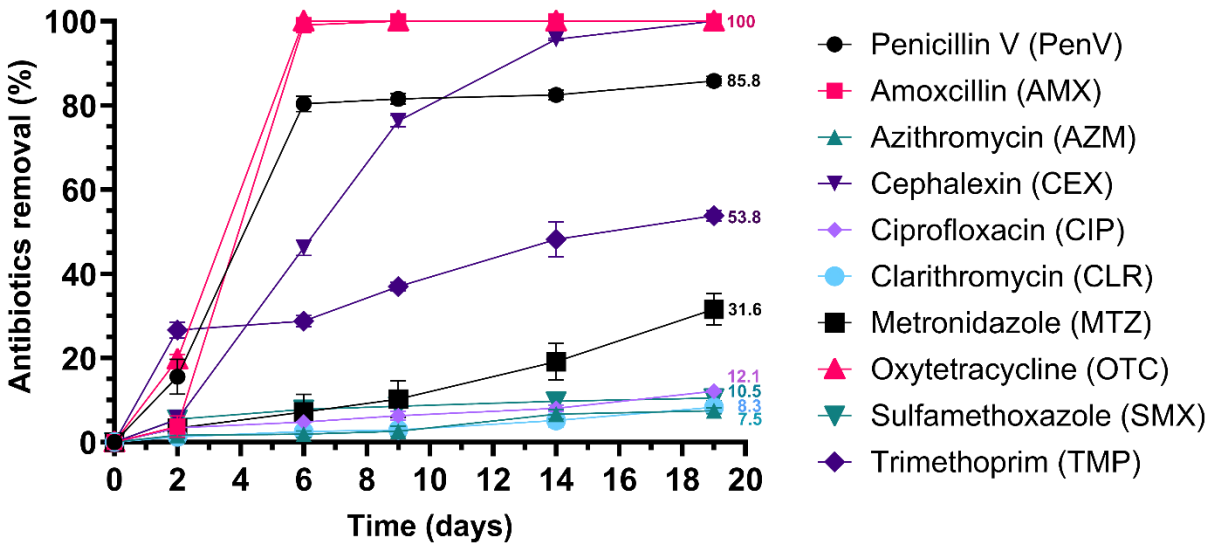

FIGURE 3: Total removal (%) of ten antibiotics by *C. sorokiniana* grown in BG11 medium.

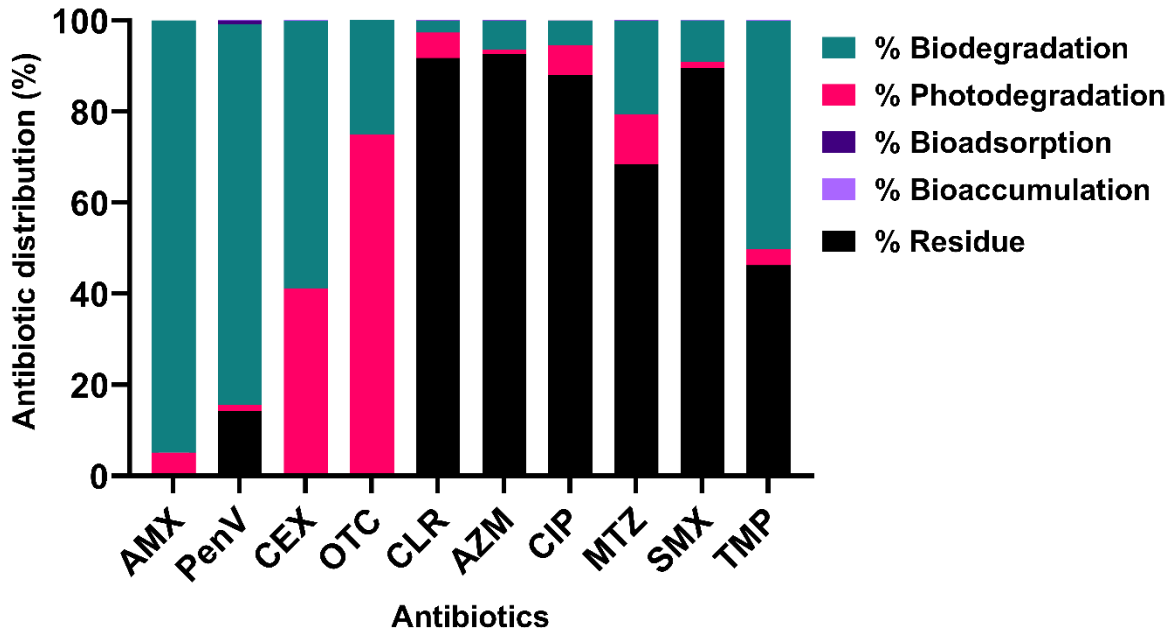

FIGURE 4: Contribution of biodegradation, photodegradation, bioadsorption, and bioaccumulation in the removal of ten selected antibiotics by *C. sorokiniana* grown in BG11 medium.

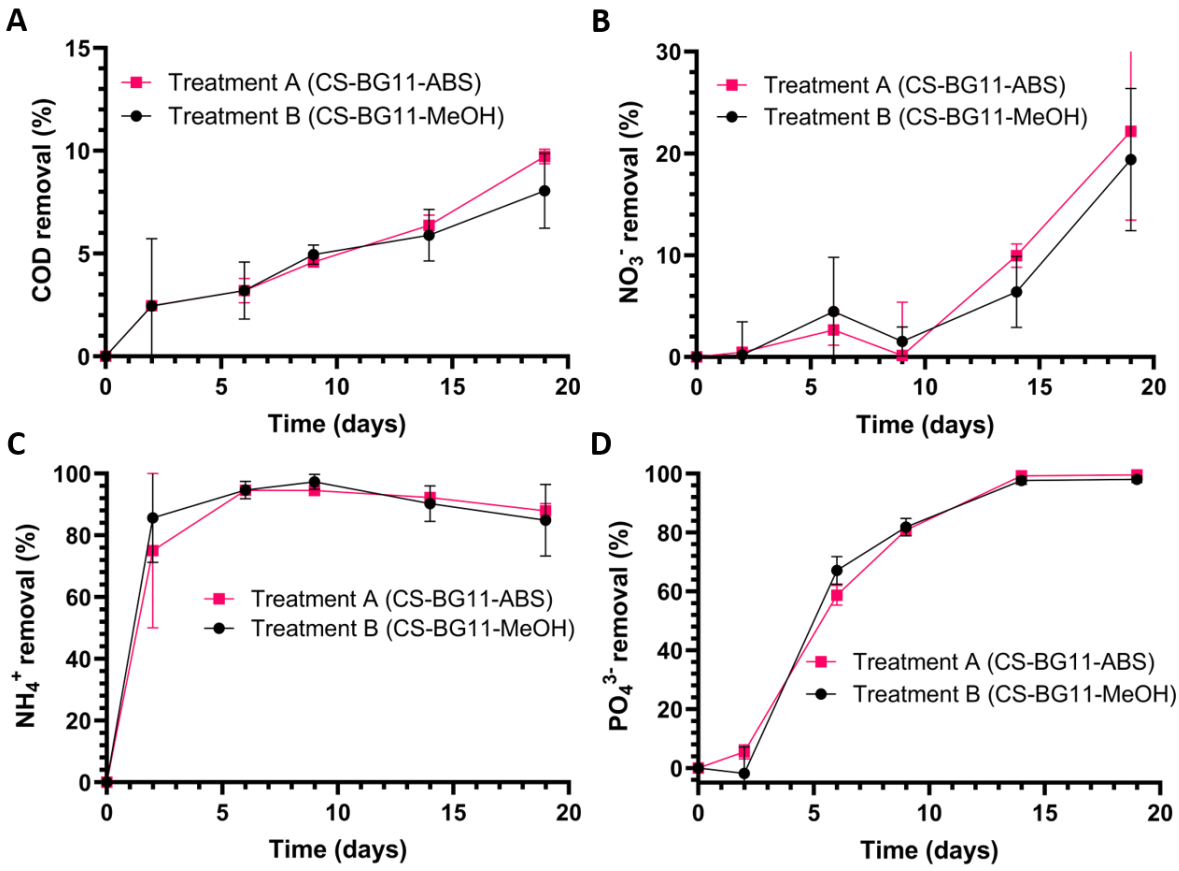

FIGURE 5: Removal of nutrients (%) by *C. sorokiniana* grown in BG11 medium in presence (treatment A, CS-BG11-ABS) and in absence (treatment B, CS-BG11-MeOH) of antibiotics. (A) chemical oxygen demand (COD), (B) nitrate, (C) ammonium, and (D) phosphate.

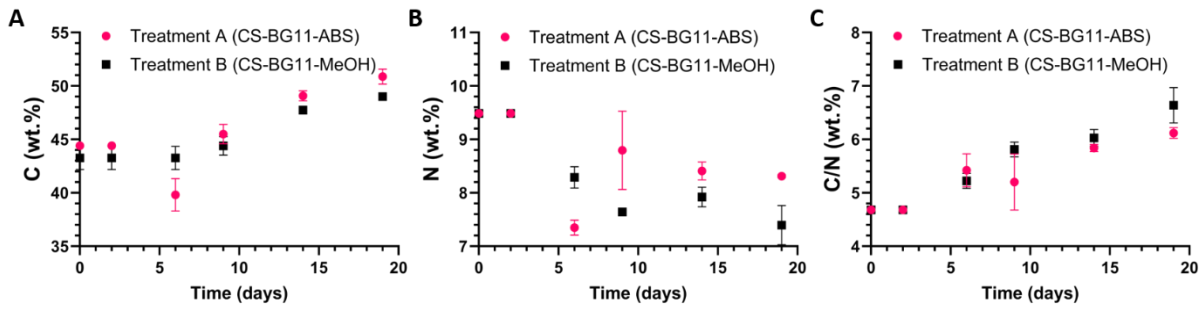

FIGURE 6: Elemental analysis of *C. sorokiniana* after 19 days of cultivation in BG11 medium in presence and absence of antibiotics. (A) C (carbon, wt. %), (B) N (nitrogen, wt. %), and (C) C/N (carbon/nitrogen ratio, wt. %).

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