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Microalgal Exposure to Human Antibiotics Triggers Similarities in Growth and Photosynthetic Responses

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Abstract

The discharge of pharmaceuticals via wastewater into the environment is a great concern due to the constant threat posed to photosynthetic organisms since they are vital for the sustenance of the aquatic food web. To compare the photosynthetic and growth responses of green algae to human antibiotics, *Raphidocelis subcapitata* and *Chlorella vulgaris* were exposed to erythromycin and sulfamethoxazole for 96 h. A much higher sensitivity was shown by *Raphidocelis* to the antibiotics. Although erythromycin was more acutely toxic to photosynthesis (EC_{50}, 24.6 µg/L; EC_{10}, 14.6 µg/L) than growth (EC_{50}, 160 µg/L; EC_{10}, 27 µg/L) in *Raphidocelis*, chronic effects in terms of EC_{10} were alike. Interestingly, sulfamethoxazole exhibited similar toxicity towards growth and photosynthesis with the acute and chronic toxicity parameters for growth (EC_{50}, >2000 µg/L; EC_{10}, 260 µg/L for *Raphidocelis*; and EC_{50}, 47,900 µg/L; EC_{10}, 19,100 µg/L for *Chlorella*) in consonance with those of photosynthesis (EC_{50}, >2000 µg/L; EC_{10}, 340 µg/L for *Raphidocelis*; and EC_{50}, 47,500 µg/L; EC_{10}, 13,400 µg/L for *Chlorella*). Growth and photosynthesis in *Raphidocelis* were strongly inhibited in this study at environmentally relevant concentrations of erythromycin. The findings from this study demonstrated that photosynthetic yield was a reliable indicator of sulfamethoxazole and erythromycin effects and thus, may be useful as an alternative approach to growth in assessing chronic toxicity in antibiotics.

Keywords

Pharmaceuticals, Antibiotics, Toxicity, Growth, Photosynthesis, Green Algae

1. Introduction

The increasing use of pharmaceuticals and their subsequent discharge into the

aquatic environment via effluents from wastewater treatment works (WWTWs) remain a source of concern [1] [2] [3] [4] [5]. Between the years 2007 and 2017, there was a 20% increase in the total number of community dispensed pharmaceutical items in Scotland [6]. The global consumption of pharmaceuticals belonging to the class antibiotics, has grown, with an estimated 65% increase between the years 2000 and 2015 and a 200% projected global consumption growth for the year 2030 [7]. Only a percentage of the parent pharmaceutical is utilised by the patient with the remainder excreted associated with urine or faeces. There are about 9000 WWTWs that the UK water industry manages [8] and depending on the pharmaceutical, the removal efficiency varies greatly in the generally aerobic processes employed in these treatment plants [9], thus allowing a percentage of the unaffected parent compound into the environment. If the sewage is not treated, then it is expected that the percentage reaching the environment increases. A 2017 United Nations (UN) world water development report stated that on average, about 30% of the municipal and industrial wastewater generated in high income countries are not treated or inadequately treated. The percentage increases to 62% in upper middle-income countries and to 72% in lower middle-income countries while in low-income countries about 92% do not undergo treatment of any type [10]. Consequently, human pharmaceutical residues in wastewater represent a global threat to non-target aquatic organisms, even at low concentrations, due to their bioactive nature and continuous infusion into the aquatic environment from WWTWs [11]. A recent study commissioned by Centre of Expertise for Waters identified eight pharmaceuticals including two antibiotics as posing a high ecotoxicological risk in Scotland's inland surface waters [12].

The green algae used in this study belong to a diverse group of aquatic photosynthesizing microscopic organisms called phytoplankton [13]. The aquatic food web is sustained by the energy obtained by phytoplankton through photosynthesis [14]. The microalgae rely on this energy to perform necessary functions including growth. In addition, about 50% - 85% of the earth's atmospheric oxygen comes from the photosynthetic activity of phytoplankton [15] [16]. Any significant reduction in their photosynthetic efficiency will not only affect their growth adversely but affect the organisms at the upper trophic levels [17]. It is therefore important to investigate the potential adverse effects of human pharmaceuticals on microalgae to evaluate the risk they pose to the aquatic environment. The freshwater green algal species, *Raphidocelis subcapitata*, have been frequently employed as model organisms to assess the toxicity of many pharmaceuticals particularly antibiotics [18]-[23]. However, lately there has been an increase in the use of the green algae, *Chlorella vulgaris*, probably due to its prevalence in freshwater systems [24] [25] [26] [27] [28].

The assessment of chemical toxicity using microalgae usually involves a growth inhibition test using the endpoint of algal biomass as described in several guidelines [29]. The method of using chlorophyll *a* fluorescence determined by Pulse Amplitude Modulation (PAM) fluorometry, although reliable for assessing her-

bicide and metal toxicity [30] [31] [32], has not been extensively used to evaluate the toxic effects of pharmaceuticals due to their modes of action [33] [34] [35] [36]. The PAM technique provides information about the photosystem II (PS II) effective quantum yield or photosynthetic fitness of the microalgae [37] [38] [39]. It can be used in a quick bioassay because it provides direct information on the photosynthetic efficiency of the algae in contrast to the standardized growth inhibition test which requires at least 72 h [40]. Escher *et al.* [33] in their measurement of photosynthesis inhibition in *D. subspicatus* using PAM obtained 24 h EC_{50} for ibuprofen, diclofenac, and carbamazepine that correlate with previously reported 72 h EC_{50} for growth inhibition of the same algal species determined according to the OECD guideline [41]. For the macrolide, tylosin, reported $EC_{50growth}$ was up to 140 times higher than effect concentrations obtained using photosynthetic efficiency as acute endpoint [35]. However, this present study intends to exploit the PAM's use as a chronic marker (≥72 h) of antibiotic effects in comparison to growth inhibition.

This study examined two individual antibiotics selected from a wide range of pharmaceuticals monitored in hospital wastewaters in the EU funded PILLs Project [42] to which this study was linked. The selection of erythromycin (ERY), and sulfamethoxazole (SUF) was based on hospital contribution, European wide usage, and persistence in the environment [42]. They have been identified as antibiotics of high risk in the aquatic environment of Europe, USA and Worldwide due to their consumption, discharge, persistence, and toxic properties [43] [44] [45] [46] [47]. This study was carried out to investigate individual toxicity of ERY and SUF to green algae, *R. subcapitata* and *C. vulgaris* by assessing and comparing effects on two physiological endpoints namely, growth and photosynthesis following 96 hr of exposure.

2. Methodology

2.1. Strain Cultivation

Unicultures of *R. subcapitata* (CCAP 278/4) and *C. vulgaris* (CCAP 211/12) purchased from Culture Collection of Algae and Protozoa (CCAP), cultivated in 500 ml conical flasks containing 200 ml sterile Jaworski's Media (JM), were maintained on a shaker (100 rpm) in a culturing apparatus at 20˚C ± 1˚C under continuous illumination in the range 30 - 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetic active radiation (PAR). To keep the cultures in an exponential growth phase, algae were aseptically transferred to fresh media every 3 - 4 days.

2.2. Chlorophyll a Fluorescence Bioassay

Test concentrations were prepared from the stock solutions of the pharmaceuticals by diluting with JM and, following preliminary range finding experiments; 12.5 - 200 µg/L of ERY; and 125 - 2000 µg/L of SUF were tested against *R. subcapitata* while *C. vulgaris* was exposed to 1560 - 25,000 µg/L ERY and 12,500 - 100,000 µg/L SUF. The samples without pharmaceuticals were used as the con-

trol. Tests were performed in sterile 3 mL glass vials containing 0.5 mL of algae inoculum and 1mL of test substance and for validation of the toxicity tests, ammonium dichromate was tested as the positive control. The tests were carried out in triplicates under axenic conditions and test vials were incubated under the same environmental conditions as the algal stock cultures for 96 h. Test vials position was randomised and changed daily [48]. To determine the stability of the pharmaceuticals in the test systems, samples were taken from the control and test vials at the beginning of the tests (without algae) and at 96 h and stored at −20˚C until chemical analysis. The chlorophyll fluorescence intensity of each treatment and control sample was determined immediately after inoculation and every 24 h using a highly sensitive dual channel ToxY-PAM chlorophyll fluorometer with a ToxYWin Software (Heinz Walz, Effeltrich, Germany) and an average of six measurements taken for each replicate. In the ToxY-PAM blue light is used for excitation and fluorescence is assessed at a wavelength above 650 nm. The (F_s) fluorescence level corresponds to the fluorescence measured shortly before the application of a saturation pulse. The measured fluorescence parameters [Fm (maximum fluorescence and Fs (minimum or steady state fluorescence)] allowed the calculation of the effective quantum yield (Y) of the linear electron transport or the efficiency of PS II photochemistry (Φ_{PSII}) which indicates the capacity of the light adapted cells to convert absorbed light energy to chemical energy. This is used as a proxy for the fitness or efficiency of photosynthetic organisms [37].

$$\Phi_{PSII} \text{ or } Y = \left(F_m - F_s\right)/F_m \quad (1)$$

2.3. Algae Growth Inhibition Test

Test microalgae were exposed to the individual test pharmaceuticals diluted in JM after range finding experiments. For *Raphidocelis*, the pharmaceutical concentrations were 1250 - 2000 µg/L SUF, and 12.5 - 200 µg/L ERY. *C. vulgaris* was exposed to 12,500 - 100,000 µg/L SUF and 6250 - 100,000 µg/L ERY. The bioassays were carried out in accordance with OECD Test Guideline 201 [49] and 50 mL conical flasks (Fisherbrand) containing 20 mL of test solution were used as test vessels. In each flask, a specified volume of algal culture in the range 1.5 to 2 mL, in exponential growth phase (3 - 4 day old) was diluted with a known volume of JM (18 to 18.5 mL), with or without pharmaceuticals, to obtain an initial cell biomass in the range 300,000 to 700,000 cells/mL for *R. subcapitata* and 1×10^6 to 1.5×10^6 cells/mL for *C. vulgaris.* Each concentration of the pharmaceutical and the control was tested in triplicates.

The tests were run for 96 h under the same standard conditions used for the inoculum culture. The positions of test flasks were randomized and changed every 24 h for uniform light distribution. Cell densities were determined every 24 h by loading a haemocytometer with 10 µL of each sample and counting under the 40 × objective, employing a Leica DM500 microscope in the bright field configuration. There were no significant changes in the pH of the control and

treatments before and after the assays. Stability of the pharmaceuticals in the test systems was determined by taking samples from the control and treatment vessels at 0, 48 and 96 h and stored at −20˚C until further analysis.

2.4. Pharmaceutical Analysis

Samples were filtered through 0.2 µm cellulose filter prior to chemical analysis. Liquid chromatography mass spectrometry (LC-MS/MS) was used to determine the actual concentrations of the pharmaceuticals. The LC system was an Agilent 1100 Series LC equipped with a Phenomenex, 5 cm Kinetex SD C18 (50 mm × 2.10 mm) column. The Bruker 3000 Esquire plus Ion Trap mass spectrometer with electrospray ionization (ESI) source was operated in positive ion mode. For each sample, 10 µl was injected using an auto-sampler. Mobile phase was 10 mmol ammonium formate (adjusted to pH = 3.5 by formic acid), and acetonitrile. The flow rate was 1.0 ml/min. All detections were performed by mass spectrometry (MS), in which the m/z transition for the drugs was as follows: 254 → 155.9 for SUF, and 734.47 → 158.1 for ERY.

2.5. Statistical Analysis

Inhibitions of growth and photosynthesis were expressed as percentages of the control. The effective concentration (EC) of each pharmaceutical that inhibited 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}) of the algal growth and photosynthesis in respect to the control and their 95% confidence intervals (CI) were determined by linear interpolation and Probit analysis using SPSS statistics package (v21, SPSS Company) [21]. The LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values for each test pharmaceuticals were determined after normality test (Shapiro-Wilk). To determine these chronic toxicity indices, a one-way analysis of variance (ANOVA) followed by Tukey and Games-Howell post hoc test were performed using SPSS statistics v26 programme. A p value < 0.05 was considered statistically significant.

Although, the assays used in this study were short-term assays and are most often considered as acute toxicity assays [50] [51], they are in principle multigenerational assays, and can also be considered as chronic toxicity tests [52] [53]. According to the EU Technical Guidance Document [54], 72-h (or longer) exposure studies with alga EC_{50} values can be considered as equivalent to short-term exposure parameters, and the NOEC values can be considered as long-term exposure parameters. In addition, the regression-based approach (EC_{10}) has been suggested as a better indicator of low toxic effect levels [55] [56].

3. Results

3.1. Effects on Algal Photosynthesis

Table 1 shows the effects of the test antibiotics on the photosynthesis of *C. vulgaris* and *R. subcapitata* within 24 to 96 h of exposure. Hormetic effects were induced in *C. vulgaris* at 24 h of exposure to SUF. However, the antibiotic

Table 1. Photosynthetic inhibition (%) in microalgae following a 96-h exposure to antibiotics.

µg/L	24 h	48 h	72 h	96 h
Chlorella				
SUF				
12,500	−10.14 ± 2.6	0.55 + 1.1	12.59 + 1.4	9.82 ± 1.8
25,000	-16.1^{a} ± 0.3	14.54 + 3.1	22.10^{a} + 0.5	19.89^{b} + 6.0
50,000	-23.4^{b} ± 3.2	27.5^{b} + 6.4	64.46^{c} + 3.1	53.33^{c} + 1.3
100,000	-26.4^{c} ± 2.2	41.6^{c} + 10.1	79.31^{c} + 7.4	96.98^{c} + 0.4
ERY				
1560	−1.91 ± 1.3	-8.71^{a} ± 0.7	−7.8 ± 1.5	−4.86 ± 1.1
3120	−1.74 ± 1.7	-7.49^{a} ± 2.0	−7.0 ± 0.8	−2.23 ± 2.5
6250	−1.52 ± 0.6	−6.38 ± 1.2	−3.21 ± 1.6	0.25 ± 1.0
12,500	−0.53 ± 0.8	−1.99 ± 2.2	−0.34 ± 3.5	3.64 ± 0.5
25,000	3.96 ± 1.5	7.22^{a} ± 1.3	9.90^{a} ± 2.6	13.03^{c} ± 3.1
Raphidocelis				
SUF				
125	−0.94 ± 1.5	-5.20^{b} + 1.5	0.17 + 1.1	4.25 ± 2.9
250	−0.06 ± 0.9	−1.26 + 0.9	0.92 + 1.3	5.65 + 2.9
500	0.81 ± 1.0	1.14 + 0.8	14.68^{c} + 1.2	17.71^{c} + 2.3
1000	3.73 ± 1.9	8.51^{c} + 0.7	19.73^{c} + 1.0	28.21^{c} + 0.8
2000	3.33 ± 0.5	9.75^{c} ± 1.1	25.66^{c} ± 1.5	37.62^{c} ± 1.8
ERY				
12.5	−2.40 ± 1.8	−0.81 ± 1.2	0.01 ± 0.9	1.23 ± 4.4
25	−2.51 ± 2.4	3.13 ± 1.1	39.58^{c} ± 1.7	51.57^{b} ± 2.3
50	0.33 ± 3.2	8.63^{a} ± 2.2	55.42^{c} ± 1.2	97.37^{c} ± 0.3
100	5.92 ± 2.3	21.64^{c} ± 1.6	65.76^{c} ± 0.9	98.92^{c} ± 0.1
200	24.20^{c} ± 3.8	33.30^{c} ± 1.6	73.74^{c} ± 1.7	99.56^{c} ± 0.1

SUF, ERY: Sulfamethoxazole, Erythromycin; Values are Mean ± SE, n = 3; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$.

caused a significant inhibition in Φ_{PSII} of *Chlorella* at 50,000 and 25,000 µg/L at 48 and 72 - 96 h respectively (Table 1). Inhibition increased from 14.5% at 48 h to 19.8% at 96 h in the 25,000 µg/L treatment. The decrease of photosynthesis in *Raphidocelis* at 2000 µg/L after 24 h was not significant ($p < 0.05$) and SUF became toxic with time with LOECs of 1000 and 500 µg/L noted at 48 and 72 - 96 h respectively (Table 1). For the 500 µg/L treatment, a rise in photosynthetic inhibition from 0.81% at 24 h to 17.7% at 96 h was observed.

In *C. vulgaris* exposure, ERY enhanced Φ_{PSII} with significant stimulation noted shortly by 48 h (up to 8.7%) at 1560 µg/L (Table 1). Significant inhibition ($p < 0.05$; 7.2% to 13%) of Φ_{PSII} in *Chlorella* was seen only at the highest tested concentration (25,000 µg/L) of ERY from 48 - 96 h. ERY was toxic at 24 h with LOEC of 200 µg/L and inhibiting Φ_{PSII} by 24%. The toxic effects of ERY on Φ_{PSII} in *Raphidocelis* increased with time and concentration-dependent significant ef-

fects were induced by 48 and 72 - 96 h (Table 1). The inhibition of Φ_{PSII} at 25 µg/L increased from 3.1% at 48 h to 51.5% at 96 h.

3.2. Effects on Algal Growth

The effects of the test antibiotics on growth in *C. vulgaris* and *R. subcapitata* within 24 to 96 h of exposure are shown in Table 2. SUF had no notable effects on growth in *R. subcapitata* until 48 h with a LOEC of 1000 µg/L (Table 2). Its toxicity increased afterwards with 72 - 96 h LOEC of 500 µg/L. A similar pattern was exhibited in *Chlorella* with lower concentrations (12,500 - 25,000 µg/L) of SUF stimulating growth within 24 h and toxic effects increased with time with LOECs of 100,000, 50,000, and 25,000 and 12,500 µg/L at 24, 48, 72, and 96 h respectively.

Table 2. Growth inhibition (%) in microalgae following a 96-h exposure to antibiotics.

µg/L	24 h	48 h	72 h	96 h
Chlorella				
SUF				
12,500	$-5.53^{b} \pm 0.8$	$-0.40 + 0.4$	$0.97 + 0.6$	$5.73^{a} \pm 0.9$
25,000	$-3.85^{a} \pm 1.0$	$2.48 + 1.0$	$6.35^{b} + 0.9$	$8.90^{b} + 1.3$
50,000	-2.03 ± 0.4	$27.73^{c} + 0.7$	$48.04^{c} + 0.6$	$61.30^{c} + 0.3$
100,000	$30.92^{c} \pm 0.9$	$62.61^{c} + 1.0$	$72.29^{c} + 0.5$	$82.50^{c} + 0.4$
ERY				
6250	$-32.8^{b} \pm 1.9$	$-19.45^{b} \pm 4.4$	$-15.13^{b} \pm 1.4$	$-9.25^{a} \pm 1.8$
12,500	$-23.2^{a} \pm 4.8$	$-11.00^{a} \pm 2.1$	-4.4 ± 0.9	-1.10 ± 0.9
25,000	-1.31 ± 2.1	2.15 ± 0.45	6.68 ± 0.7	$12.10^{a} \pm 1.9$
50,000	-0.40 ± 1.2	$16.80^{b} \pm 1.0$	$25.60^{c} \pm 2.9$	$38.35^{c} \pm 2.4$
100,000	$19.90^{a} \pm 4.8$	$33.35^{c} \pm 1.1$	$42.55^{c} \pm 1.4$	$53.00^{c} \pm 2.0$
Raphidocelis				
SUF				
125	-0.56 ± 0.2	$-5.40 + 0.8$	$0.03 + 1.4$	3.48 ± 1.6
250	-4.4 ± 0.3	$-1.53 + 2.4$	$0.30 + 2.3$	$8.63 + 2.7$
500	0.98 ± 0.7	$0.25 + 2.7$	$15.00^{c} + 1.5$	$17.26^{c} + 1.2$
1000	1.49 ± 0.9	$9.70^{a} + 1.1$	$19.96^{c} + 1.8$	$32.56^{c} + 2.9$
2000	1.86 ± 1.1	$10.73^{a} \pm 1.1$	$28.26^{c} \pm 0.9$	$39.36^{c} \pm 2.8$
ERY				
12.5	$-20.8^{c} \pm 2.2$	-9.00 ± 2.4	-2.16 ± 0.9	1.75 ± 0.5
25	-10.34 ± 3.6	-1.15 ± 2.7	2.75 ± 0.8	$13.63^{c} \pm 1.7$
50	-0.56 ± 1.1	2.86 ± 2.7	$11.02^{a} \pm 2.4$	$20.28^{c} \pm 0.8$
100	1.67 ± 1.0	$14.85^{a} \pm 2.7$	$28.02^{c} \pm 1.2$	$35.24^{c} \pm 0.6$
200	10.68 ± 1.5	$26.71^{c} \pm 2.9$	$47.63^{c} \pm 1.6$	$57.13^{c} \pm 2.0$

SUF, ERY: Sulfamethoxazole, Erythromycin; Values are Mean ± SE, n = 3; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$.

For *C. vulgaris*, low ERY concentrations (6250 & 12,500 µg/L) exerted stimulatory effects up to 48 h. ERY toxicity towards growth yield in *Chlorella* increased with time and LOECs of 100,000, 50,000, and 25,000 µg/L were observed within 24, 48 - 72, and 96 h respectively. By 24 h of exposure, ERY had no significant inhibitory effects on growth in *Raphidocelis* but rather caused a significant stimulation at 12.5 µg/L. It became toxic overtime with LOECs of 100, 50 and 25 µg/L observed at 48, 72, and 96 h (Table 2).

3.3. Toxicity Ranking

The 96 h EC_{10}, EC_{20}, EC_{50} as well as LOEC and NOEC values obtained for the various pharmaceuticals tested against photosynthetic efficiency and growth in the algal species are shown in Table 3. In *C. vulgaris*, after 96 h exposure period, the acute toxicity ranking of the pharmaceuticals towards Φ_{PSII} based on EC_{50} is SUF (EC_{50}, 47,500 µg/L) > ERY (EC_{50}, >25,000 µg/L) and the chronic toxicity ranking based on NOEC or EC_{10} is SUF (NOEC, 12,500 µg/L; EC_{10}, 13,400 µg/L) > ERY (NOEC, 12,500 µg/L; EC_{10}, 20,900 µg/L). For *R. subcapitata*, acute toxicity to photosynthesis is ranked in the following order: ERY (EC_{50}, 24.6 µg/L) > SUF (EC_{50}, > 2000 µg/L); and chronic toxicity in the order, ERY (NOEC, 12.5 µg/L; EC_{10}, 14.6 µg/L) >SUF (NOEC, 250 µg/L; EC_{10}, 340 µg/L).

The most acutely and chronically toxic compound to the growth of *R. subcapitata* was ERY (EC_{50}, 160 µg/L; NOEC, 12.5 µg/L) followed by SUF (EC_{50}, > 2000 µg/L; NOEC, 250 µg/L). For *C. vulgaris* exposure, in terms of acute and chronic toxicity, SUF was the most toxic compound (EC_{50}, 47,900 µg/L; NOEC, <12,500 µg/L) followed by ERY (EC_{50}, 84,100 µg/L; NOEC, 12,500 µg/L). Since effects on the yield of photosynthesis was investigated in this study, the inhibitory

Table 3. Toxicity indices (µg/L) for test pharmaceuticals after 96 h.

Drug/Endpoint	EC_{10}/EC_{20}	EC_{50}	LOEC/NOEC
Raphidocelis			
SUF			
Growth	260/590	>2000	500/250
Photosynthesis	340/610	>2000	500/250
ERY			
Growth	27/50	160	25/12.5
Photosynthesis	14.6/17	24.6	25/12.5
Chlorella			
SUF			
Growth	19,100/26,200	47,900	12,500/<12,500
Photosynthesis	13,400/25,100	47,500	25,000/12,500
ERY			
Growth	19,200/31,900	84,100	25,000/12,500
Photosynthesis	20,960/>25,000	>25,000	25,000/12,500

SUF: Sulfamethoxazole, ERY: Erythromycin.

concentration for growth yield was selected above growth rate as the endpoint of comparison to photosynthetic yield. The Commission Directive 67/548/EEC classifies substances according to their EC_{50} values for aquatic organisms as follows: very toxic (EC_{50}, ≤ 1000 μg/L), toxic (EC_{50}, > 1000 to ≤ 10,000 μg/L), and harmful (EC_{50}, > 10,000 to ≤ 100,000 μg/L) (European Commission, 2001). Substances with an EC_{50} > 100,000 μg/L would not be classified. This classification however depends on the test species and the endpoint used. For both phenotypic endpoints, in this present study with *R. subcapitata*, ERY was classified as very toxic, and SUF classified as toxic, whereas, with *C. vulgaris*, both antibiotics were classified as harmful.

3.4. Analysis of Exposure Concentrations

In both chlorophyll fluorescence and growth inhibition assays, almost all the pharmaceutical concentrations were within 80% - 120% of the nominal concentrations [49]. Concentrations did not fall under 83% of the nominal values. Since no significant differences were found between the nominal and measured exposure concentrations of the test pharmaceuticals, the nominal concentrations were used for data analyses throughout this study.

4. Discussion

The higher sensitivity shown in both the photosynthetic and growth inhibition assays by *R. subcapitata* to the antibiotics, compared to *C. vulgaris,* agrees with findings from previous work evaluating the toxicity of antimicrobial agents on these species [24]. Kasai and Hatakeyama [57] and Kasai *et al.* [58] also reported the same tendency in the sensitivity of these algae to several herbicides, ascribing the higher resistance in *C. vulgaris* partly to its thicker cell wall and superior enzyme activities. The growth and photosynthetic stimulatory responses of the test algae to low doses of pharmaceutical stress observed in this study, a phenomenon known as hormesis has been widely reported in plants and algae [59] [60].

The toxic effects of SUF on growth in this study agreed with previous report by Nie *et al.* [61]. In addition, the LOEC (500 μg/L) obtained for *Raphidocelis* using the photosynthetic endpoint in this study correlated with the value reported by Liu *et al.* [62]. The toxic effects of SUF on *Chlorella* have been scarcely investigated [25] [63], and a much higher EC_{50} (18×) was obtained for SUF in this study. The discrepancy in results may be attributed to the differences in assay techniques, culturing conditions and algal media used.

SUF exhibited similar toxicity towards growth and photosynthesis in *Raphidocelis* and *Chlorella* in this study. It is quite intriguing that despite the vast differences in the techniques used, the toxicity parameters (EC_{10}, EC_{20} and EC_{50}) for growth yield are in consonance with those of the photosynthetic yield for each of the microalgae following SUF exposure (**Table 3**). This suggests the possibility of a similar mechanism of toxicity being employed by the antibiotic against both endpoints. Sulfonamides are known to inhibit tetrahydrofolic acid synthesis in bacteria and some eukaryotes via the inhibition of dihydropterinic acid synthe-

tase, and the growth inhibitory effect of SUF on green algae is attributed to this mechanism of action [24]. Interestingly, several studies in the past have argued that pteridines including tetrahydrofolate may be involved in the stimulation of photophosphorylation in autotrophs [64] [65] [66]. Consequently, the possible participation of tetrahydrofolic acid in the photosynthetic process is supported by the strong link and the similarities in the toxic responses obtained for the endpoints of algal growth and photosynthesis in this study. This indicates that the photosynthetic yield may be used as a reliable alternative for growth yield in measuring the toxicity of sulfonamides.

The NOEC values (<1000 μg/L) obtained for SUF using both endpoints, particularly against *Raphidocelis* suggest that the sulfonamide can cause a long-term adverse effect in the environment [67]. Although, the toxic levels obtained for SUF in this study are above present environmentally realistic levels, its coexistence with other antibiotics or contaminants that are highly toxic to green algae could cause synergistic effects and pose a major risk to algal growth and photosynthesis in the aquatic environment. Further studies investigating its mixture toxicity is therefore recommended.

ERY presented the highest threat (both short term and long term) to the growth of *R. subcapitata* with 96 h EC_{50} of 167 μg/L and LOECs of 50 μg/L respectively. Macrolides inhibit protein synthesis by binding to the 23S rRNA molecule in the 50S subunit of the bacterial ribosome. ERY's high toxicity to growth in *R. subcapitata* has been widely reported with EC_{50} values ranging between 20 - 350 μg/L [24] [53] [18] [61] [47]. An EC_{50} of 33,800 μg/L was reported for *C. vulgaris* [24] following ERY exposure which is lower than the value reported in this study.

ERY was more acutely toxic to photosynthesis than growth in *Raphidocelis* with a lower EC_{50} value of 24.6 μg/L. However, ERY elicited similar chronic and low toxic responses from growth yield and photosynthetic yield in both algal species (**Table 3**). This correlation further corroborates the argument of Liu *et al.* [62] that the toxic effects of certain antibiotics to green algae could be associated with the retardation of metabolism in the chloroplast facilitating a disturbance in the function of photosynthetic apparatus and eventually inhibiting cell growth. Although, the green alga is a non-target organism for antimicrobials, and unlike herbicides, none of the test antibiotics are specifically designed to affect the photosynthetic apparatus, the relatively high toxicity exerted by ERY towards photosynthetic activity in *R. subcapitata* in this study could partly be as a result of the cyanobacterial nature of the replicating, transcriptional and translational system of chloroplasts which make these plastids susceptible as potential antibiotic targets [18] [68].

ERY has been detected up to a concentration of 90 μg/L (higher than the LOEC or EC_{20} for growth in this study) in surface water [43] and was also detected in a river body in Scotland up to 24.2 μg/L [69] which is close to $EC_{10growth}$ and equivalent to $EC_{50photosynthesis}$ in this present study. This shows that ERY will adversely affect growth and photosynthesis of *R. subcapitata* in the ecosystem at

some of the concentrations at which they occur in the environment. The macrolide drugs were placed on the "watch list" of chemicals monitored under the Water Framework Directive (WFD) because of their high ecotoxicity as well as the risk they posed to human health through the development or selection of antibiotic resistant bacteria in the environment [70]. The results obtained in this study further indicate that the release of ERY into the aquatic environment after therapeutic use poses a significant long-term ecotoxicological risk to the microalgal community. In this study, photosynthesis was a more sensitive parameter for the macrolide, ERY probably due to its mode of action and it would likely be a reliable endpoint for antibiotics designed to target bacterial protein synthesis and/or DNA replication. Majority of the toxicity studies using algae are 3 - 4 days exposures [13] and chronic studies (≥7 days exposure) investigating the molecular pathways or mechanisms of toxic effects of antibiotics on microalgae should be carried out as this is not fully understood.

5. Conclusion

ERY was highly toxic to *R. subcapitata*, strongly inhibiting growth and photosynthesis in this study at environmentally relevant concentrations. Consequently, their release into the environment via WWTPs after normal therapeutic continues to pose a substantial risk to the phytoplankton community. The findings from this study showed that photosynthetic yield was a reliable indicator of SUF and ERY effects and thus, may be used as an alternative approach to growth in assessing toxicity. Since photosynthesis was found to be more sensitive to the ERY than growth in *R. subcapitata* partly due to the evolutionary conservation of the bacterial genome/translational system and their effect pathways in the chloroplasts of green algae [68], it can be implied that the endpoint will be useful in assessing chronic toxicity in antibiotics that are known to interfere with bacterial protein synthesis or DNA replication. It will be therefore useful to employ the photosynthetic efficiency endpoint as a tool in evaluating the chronic effects of a wide range of antibiotics and their mixtures, based on their modes of action, in a variety of freshwater green algae at different levels of biological continuum.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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