Pharmaceuticals removal and nutrient recovery from wastewaters by *Chlamydomonas acidophila*

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1 PHARMACEUTICALS REMOVAL AND NUTRIENT RECOVERY FROM
2 WASTEWATERS BY *CHLAMYDOMONAS ACIDOPHILA*
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Abstract

Wastewater treatment plants are a major source of human pharmaceutical residues (PR) in the aquatic environment, which are considered to be of emerging concern. The microalga *Chlamydomonas acidophila* has shown to be a promising technology to recover the nutrients from wastewater and therefore, the aim of this study was to evaluate the ability of this species to also remove PRs commonly present in wastewaters (atenolol, caffeine, carbamazepine, clarithromycin, erythromycin, lidocaine, propranolol and simvastatin). Batch assays were carried out (50mL, 90rpm, 43 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 22°C) comprising the wastewater with the pharmaceutical mixture at three concentrations: CHigh, CModerate and CEnv. The results demonstrated that this microalga does not seem to be affected by pharmaceuticals in wastewater at concentrations well above those detected in urban effluents; additionally it is able to degrade the antibiotics erythromycin and clarithromycin better than other microalgae species and enhances their removal from wastewater by 93 - 65% and 64 - 50% respectively. Furthermore, it exhibited a high assimilation of ammonium and phosphates reaching values of around 9 $\text{mgNH}_4 \text{L}^{-1}\text{d}^{-1}$ and 3 $\text{mgPO}_4 \text{L}^{-1}\text{d}^{-1}$ compared to other species. Therefore, *Chlamydomonas acidophila* appears to be a promising agent for urban wastewater treatment.

Keywords: Microalgae; antibiotics; urban wastewaters; biological treatment; nutrients recovery.

1. Introduction

In recent years there has been increasing scientific and regulatory attention towards emerging micro-pollutants such as pharmaceutical residues in the aquatic environment. Pharmaceutical residues (PR) can originate from agricultural activities, hospital effluents, industrial wastes and domestic wastes. They are typically found at very low concentrations in the environment and are unlikely to affect human health but could cause chronic exposure damage to aquatic organisms [1,2]. In addition, the continually growing and ageing population and improving quality of life worldwide means that pharmaceuticals consumption is set to increase, necessitating continual review of risk assessments [3].

Following human consumption, PR are generally excreted into the sewer and subsequently reach wastewater treatment plants (WWTPs), where they are not fully removed [4,5]. Thus, WWTP are a major source of human PR in the aquatic environment, at least in regions where most properties are connected to the sewer.

Reflecting growing concerns regarding the environmental impact of PR, the European Commission added the macrolide antibiotics (erythromycin, clarithromycin and azithromycin) to its 'Watch List' in 2015. This list, first introduced in 2013, requires union-wide monitoring of substances that are suspected of posing an environmental risk but for which insufficient monitoring data is available to determine their actual environmental risk. Two more antibiotics, ciprofloxacin and amoxicillin, were added in 2018 [6]. As pharmaceutical substances are designed to be bio-active, they can have very low predicted no-effect concentrations above which they may pose ecotoxicological risks. Their continuous input leads to pseudo-persistence in the environment. Additionally, the presence of five antibiotics in the new Watch List aligns with the European One Health Action Plan against Antimicrobial Resistance (AMR),

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58 which supports the use of the Watch List to “improve knowledge of the occurrence and
59 spread of antimicrobials in the environment”.

60 Nearly a century after the discovery of penicillin, the global population is experiencing
61 widespread treatment failure from previously treatable bacterial infections. This crisis
62 stems from chronically poor antibiotic stewardship in the clinical and veterinary/animal
63 husbandry setting, which is further compounded by ubiquitous environmental pollution
64 of antibiotics and antibiotic resistant genes from industry, sewage and manure [7].

65 Upon consumption, most antibiotics are partly metabolised, with a fraction of the consumed
66 amount excreted into the sewer as parent compound. These residues, along with any
67 metabolites, thus reaches WWTP, where some are resistant to conventional physico-chemical
68 and biological processes and are only partially removed [8]. Subsequently, antibiotics will
69 enter the receiving river [9], from which they can exert a selective pressure for resistant
70 genes and potentially give rise to AMR. In fact, the conditions in conventional
71 biological wastewater treatment can – in themselves - induce increased development of
72 antibiotic resistance due to the co-existence of various environmental stressors and pre-
73 existing antibiotic resistance genes [10].

74 Although several advanced treatment technologies are available for removing PRs, the
75 low removal efficiencies or financial and technical limitation of the application of these
76 technologies in wastewater treatment continue to drive research for better treatment
77 strategies for the removal of these PRs [2].

78 Among new developments in biological wastewater treatment, microalgae are used
79 around the world for the removal of nutrients from different waste effluents.

80 *Chlamydomonas acidophila* have been shown as promising agents for the removal and
81 recovery of nutrients from effluents in North-West Europe and similar climatic regions,
82 as they can operate at low temperatures and low light intensity and is able to tolerate
83 very high ammonium concentrations of up to 1000 mg NH₄-N L⁻¹ [11]. Additionally,

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84 mixotrophic algae, such as *C. acidophila*, have an additional benefit applicable in
85 wastewater treatment, in that they can switch their metabolism between autotrophic and
86 heterotrophic mode depending on the availability of carbon sources and nutrients in the
87 surrounding environment, which provides them a great flexibility to survive and thrive
88 in variable and extreme conditions [2,12–14]. However, no information is available
89 regarding their potential for removing pharmaceuticals, or on the impact of
90 pharmaceuticals on their growth and functioning.

91 This paper investigates the use of *Chlamydomonas acidophila* in addressing three
92 concurrent needs to develop improved wastewater treatment techniques: removal of
93 nutrients, pharmaceuticals generally, and antibiotics specifically. Therefore, the aims of
94 this study were to investigate the growth and nutrient consumption of *Chlamydomonas*
95 *acidophila* in an anaerobically pre-treated wastewater containing different
96 pharmaceuticals and to determine the ability of this species to remove these micro-
97 pollutants. The anaerobic effluent, as may arise from digestate of sewage sludge, was
98 chosen due to the twin problem of high nutrient concentration (AD does not remove
99 nutrients) and pharmaceutical residues including a range of antibiotic substances.

100 **2. Materials and methods**

101 *2.1. Microorganisms and culture conditions*

102 The strain of *Chlamydomonas acidophila* used in the study was obtained from the
103 Göttingen collection of algae for culture (Sammlung von Algenkulturen Göttingen
104 [SAG], Germany). Cells used for the inoculum were grown in bottles of constant
105 working volume 1 L (ensured by addition of distilled water to replace water lost by
106 evaporation), which was maintained at room temperature and light conditions, bubbled
107 with air.

The microalgae were maintained by periodic transfer to a standard medium (SM) containing the following ingredients: $(\text{NH}_4)_2\text{SO}_4$ (1000 mg L⁻¹), K_2HPO_4 (200 mg L⁻¹), MgSO_4 (20 mg L⁻¹), Na_2EDTA (130 mg L⁻¹), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (80 mg L⁻¹), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.18 mg L⁻¹), ZnCl_2 (0.052 mg L⁻¹), and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.063 mg L⁻¹). The cells were harvested and separated from the media by centrifugation at 2500 rpm for 3 min. The separated cells were washed 3 times with distilled water and the centrifugation step was repeated to remove all culture medium prior to use in the different assays.

2.2. Wastewater

Among the processes used for wastewater treatment (WWT), anaerobic processes have the advantage of reducing the organic matter of municipal and industrial wastewaters producing energy at the same time [15]. Furthermore, complex organic nitrogen and phosphorus compounds are mineralized to $\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$ during AD [16], which are key nutrients for controlled (remediation processes) and uncontrolled (eutrophication) growth of microalgae. Given the extensive use of anaerobic digestion associated with WWT in the UK and the drive to reduce nutrient pollution of water bodies via various directives [Urban Wastewater Treatment (91/271/EEC) [17]; Nitrates (91/676/EEC) [18] and Water Framework (2000/60/EC) [19] Directives] nutrient rich anaerobically treated wastewater is an important waste stream both for nutrient and pharmaceutical pollution. Therefore, for the present study, anaerobically treated wastewater was used in the assays carried out.

In order to provide consistent conditions for these experiments, artificial wastewater effluent was generated in the laboratory by anaerobic digestion of a synthetic wastewater adapted from the Organization for Economic Cooperation and Development [20], to simulate the constituents of typical wastewater: tryptone 350mg L⁻¹, meat extract 110mg L⁻¹, K_2HPO_4 28mg L⁻¹, NaCl 7mg L⁻¹, CaCl_2 3mg L⁻¹ and MgSO_4 2mg

133 L⁻¹. The synthetic wastewater was digested in a 6 L volume completely mixed anaerobic
134 bioreactor maintained at a temperature of 35°C, loading rate of 0.9 g L⁻¹ d⁻¹ of volatile
135 solids (VS) and a hydraulic retention time (HRT) of 4 days. In order to retain the solids
136 in the system, stirring was stopped 15 min before feeding and effluent extraction, so that
137 the solids could settle and effluent be removed from the liquid phase. The effluent was
138 stored at 4°C prior to the assays.

139 Chemical Oxygen Demand (COD), pH, NH₄ and PO₄ contents of the anaerobically
140 treated effluent were around 250 mg/L, 6.8, 110 mg L⁻¹ and 30 mg L⁻¹ respectively.

141 *2.4. Experimental set-up*

142 *2.4.1. Removal and influence of the different pharmaceuticals on growth and nutrient* 143 *recovery of C. acidophila*

144 Batch assays were carried out in an orbital incubator shaker, in which cultures of 50 mL
145 were stirred at 90 rpm in 250 mL glass Erlenmeyer flasks at 22°C. The surface of the
146 flasks was continuously illuminated, at a rate of approximately 43 μmol photons m⁻² s⁻¹
147 by LED lights.

148 Eight different PR were selected based on consumption patterns, ecotoxicity,
149 therapeutic class, regulatory relevance, and availability of an analytical method in the
150 laboratory, and studied at different concentrations (Table 1).

151 Reported concentrations of these PRs in urban wastewater were gathered and, in a first
152 phase (Phase 1), in order to stress the system, the concentrations added to the
153 wastewater were increased 1000 times from both the lowest and the highest
154 concentrations found in literature (CModerate and CHigh, respectively). A second batch
155 test (Phase 2) was also carried out to study the removal and influence of these PRs
156 under environmentally relevant concentrations, where the highest reported
157 concentrations were added to the wastewater (CEnv).

TABLE 1

All samples were incubated in triplicate: Standard growing medium with microalgae (SM), a Control sample (the wastewater with microalgae) (WW) and samples comprising the wastewater with the pharmaceutical mixtures at the 3 concentrations (CHigh, CModerate and CEnv) with and without microalgae. Abiotic batches were set up to test for any non-biological micropollutant removal or degradation. Furthermore, because PRs were dissolved in methanol before their addition to the wastewater and this compound might affect the microalgae behaviour, a sample containing wastewater, microalgae and methanol (the same volume as added in CHigh) was included (WW+Met).

The flasks containing microalgae were inoculated from the seed culture with 2.5×10^6 cells mL^{-1} . Cell numbers, and PR, ammonium and phosphate concentrations were measured every 2 – 3 days in each sample during the incubation period (14 days). Two subsamples were collected each sampling day. Both samples were centrifuged at 13,000 rpm for 3 min and one was stored at 4 °C until nutrients analysis and the second one was stored at -20 °C until PR analysis.

2.4.2. Subsequent experiments on the removal of the antibiotics

Because significant removal of the antibiotics was observed during the initial batch experiments, the antibiotics erythromycin and clarithromycin were studied separately using standard growing media (SM), in order to evaluate their removal without any interference from other PR or wastewater constituents. Assays were carried out under the same operational conditions as the main experiments, in triplicate and over an incubation period of 7 days. The two antibiotics were studied separately, in a mix of SM and under the environmentally relevant concentrations of 2.3 and 8 $\mu\text{g L}^{-1}$ of erythromycin and clarithromycin, respectively.

2.4. Chemical analysis

The pH, total solids (TS) content and VS content were determined according to the Standard Methods for the Examination of Water and Wastewater [36]. The cells concentration in the assays was measured using Celeromics Technologies S.L Micro Counter®. Determination of NH_4^+ in the samples was carried out according to a colorimetric method the colorimetric method based on the Nessler protocol [37]. The standard method 4500 P-E was used for PO_4^{3-} analysis [36]. Samples were centrifuged at 13,000 rpm for 3 min before NH_4^+ and PO_4^{3-} analysis.

2.5. Pharmaceuticals analysis

The mass spectrometer used for the pharmaceuticals analysis was a Thermo Scientific Q-exactive Orbitrap mass spectrometer, fitted with a Dionex Ultimate 3000 RS pump, Dionex Ultimate 3000 RS autosampler (temperature controlled at 10°C) and Dionex Ultimate 3000 RS column compartment (temperature controlled at 30°C). The operating software was Chromeleon, Xcalibur and Tracefinder. In both positive and negative ionisation mode the electrospray conditions were sheath gas 45 arbitrary units, auxiliary gas 10 arbitrary units, capillary and auxiliary gas temperature 300 °C. The spray voltage was set at 3.5 kV and -4.5 kV in positive and negative mode respectively. The pharmaceuticals were detected in positive or negative mode using parallel reaction monitoring using the transitions described in “Supplementary information”. The mobile phase for the LC separation was A = acetonitrile, B = 10mmol ammonium formate in water adjusted to pH 3.5 with formic acid) The gradient LC conditions were: 99%B for 1 minute then up to 70% B over 1 minute. Maintained at 70% B for 5 minutes then up to 1% B over a further 1 minute. The gradient was maintained at 1% B for a further 3 minutes, back to 99% B over 1 minute and re-equilibrated with 99% B for 8 minutes. The flow was 0.2 mL min⁻¹ and the injection volume was 10 µL.

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208 Samples were centrifuged at 13,000 rpm for 3 min and the supernatant was filtered
209 through a 0.2 μm nylon syringe filter ready for PR analysis in the liquid phase.
210 Once the supernatant was removed, for the analysis of the antibiotics adsorbed to the
211 cell surface, 0.5 mL water was added to the pellet which was vortexed and centrifuged
212 again before adding a further 0.5 mL methanol and repeating the procedure of vortexing
213 and centrifugation. The pellet remaining after the methanol wash was re-suspended in
214 methanol (0.5 mL). After centrifugation the supernatant was removed, dried under a
215 stream of nitrogen at room temperature, re-constituted in 0.5 ml of acetonitrile:water
216 (30:70) and filtered through a 0.2 μm nylon syringe filter ready for analysis.
217 The concentration of antibiotics accumulated in the cells were analysed by a disruption
218 of the cells adding glass beads to the remaining pellet and mixed at 6.5 m s^{-1} for 60
219 seconds in a bead beater; this was repeated another 9 times. After centrifugation the
220 supernatant was removed and prepared for analysis following the same procedure as
221 before.

34 222 *2.6. Statistical analysis*

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223 Tests for significant differences between the samples in the study parameters were
224 performed using SPSS 15.0 for Windows. One-way ANOVA was used when the data
225 were not compared over time. After checking the data for homocedasticity and normal
226 distribution of the variances, the LSD test was used for multiple average comparisons
227 and for detection of any differences between pairs of variables. Differences were
228 considered significant at $p < 0.05$.

51 229 **3. Results and discussion**

52 230 *3.1. Removal of pharmaceuticals during the incubation of C. acidophila*

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231 Removal rates differed significantly depending on the pharmaceutical studied. On the
232 one hand, the concentration of some compounds like caffeine and simvastatin decreased

abruptly in the first days of incubation at the 3 different concentrations (CHigh,
 CModerate and CEnv) regardless of the presence of the microalgae. On the other hand,
 compounds like carbamazepine and lidocaine were more persistent and their removal
 was non-existent (Figure 1).
 The high caffeine elimination in the samples coincides with the results obtained in other
 studies. Some authors concluded that caffeine is not photodegradable and therefore,
 biodegradation seem to be the significant means of caffeine removal [38,39]. According
 to Matamoros et al. [38], caffeine is removed more easily by the combined presence of
 microalgae and wastewater because of the presence of bacteria in the wastewater and
 the activity of microalgae. In the present study, the decrease of caffeine concentration
 was so abrupt in all the samples that we could not observe any differences between
 them. Regarding simvastatin, in CModerate, the initial concentration added in the
 samples was 900 µg L⁻¹ but the measured concentration at t₀ was 2 µg L⁻¹ (Figure 1).
 This similar low concentration for simvastatin at t₀ was also observed for the CEnv and
 CHigh (data not shown). Further investigation of the literature reported simvastatin to
 be unstable under aqueous conditions and readily forms an acid moiety from the
 lactone. This is minimised between pH 4 and 5 [40] but not eliminated. Despite the
 samples being frozen after removal from the reactor they still contain water and hence
 simvastatin will still degrade. This would explain its low initial concentration and
 complete removal from the media.

FIGURE 1

The removal patterns of carbamazepine and lidocaine were similar at the 3 different
 concentrations and in samples with and without microalgae (final concentrations were
 not significantly different), which means that they are resistant to biodegradation under
 aerobic conditions. Furthermore, in agreement with previously published studies, the

photodegradation, biodegradation and sorption of these compounds seem to be negligible [32,41,42]. Therefore, these compounds were not removed.

With regard to atenolol and propranolol, under aerobic conditions, the speciation of these ionisable compounds changes influencing their photolysis, leading to their removal due to phototransformation [43]. In the present study, the low pH in the medium might thus be the reason why very little removal of these compounds was observed.

FIGURE 2

Previous studies have found, in addition to biotransformation, photolysis appears to be a major removal mechanism in algal treatment systems; unlike most other biological treatment processes, algal treatment increases the removal of the light-sensitive micropollutants due to the illumination of the system [42].

In the present study, regardless of their initial concentration (CHigh1000, CLow1000 and CEnv), removals of caffeine, simvastatin, carbamazepine, lidocaine, atenolol and propranolol were similar in all cases, which seems to be attributed to bio- or phototransformation. Limited previous studies have found that algal strains can induce biotransformation or biodegradation of chemicals [44].

By contrast, the antibiotics erythromycin and clarithromycin appeared to be affected by the presence of the microalgae in the media, where their removal was higher than in samples without microalgae for CEnv and CModerate concentrations (Figure 2). At the highest concentrations of pharmaceuticals (CHigh), the microalgae were strongly inhibited (see 3.2.), and therefore, no significant differences in these antibiotics removals were observed in samples with or without microalgae.

Removal of erythromycin was 93% and 65% higher and removal of clarithromycin 50 and 64% higher in the samples with microalgae, compared to samples without

microalgae (at CEnv and CModerate respectively). Therefore, it seems that *Chlamydomonas acidophila* enhanced erythromycin and clarithromycin removals in wastewater. These values are slightly higher for erythromycin and similar for clarithromycin than the improved removals by microalgae reported by Zhou et al. [44] using *C. reinhardtii*, *S. obliquus*, *C. pyrenoidosa* and *C. vulgaris*, and by Hom-Diaz et al. [45] using a local non-identified microalgae community.

The antibiotics experiments were repeated in order to better understand the mechanisms involved. Removal and effect of erythromycin and clarithromycin were monitored in isolation from those of the other PRs and using growing media instead of wastewater to avoid any interference. The same patterns were observed, where *C. acidophila* removed around a 60% and 80% more erythromycin and clarithromycin respectively compared to samples without microalgae. Therefore, it can be concluded that the presence of microalgae was the cause of these antibiotics' removals.

Under acid conditions erythromycin converts rapidly to two metabolites: anhydroerythromycin and erythromycin enol ether. These have the same chemical formula and cannot be distinguished from each other by MS and were measured as one entity. However, both are known to be biologically inactive [46]. The metabolites were found in concentrations inversely proportional to erythromycin removals in all the samples. Therefore, it seems that the microalgae enable a faster conversion of erythromycin to the metabolite forms reducing antibacterial activity and thus, decreasing the antimicrobial selection pressure, which is the on-going continual exposure to antibiotics at sub-lethal or near lethal level and is the primary driver for AMR development and maintenance.

One of the degradation products from clarithromycin found in the samples was de(cladinosyl) clarithromycin, which is known to be pharmacologically active. This

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308 compound was observed in comparable amounts to clarithromycin (data not shown),
309 however, it remained constant in all the samples during the incubation period. In the
310 present study, even if the microalgae were not able to remove de(cladinosyl)
311 clarithromycin from wastewater, they did remove the parent compound, clarithromycin,
312 decreasing therefore, the overall antimicrobial selection pressure.
313 The mechanisms of pharmaceuticals removal by microalgae can include bioadsorption,
314 bioaccumulation and intracellular and extracellular biodegradation. Microalgae
315 bioadsorption of PRs is extracellular, therefore, the sorption process varies significantly
316 according to the hydrophobicity, structure, and functional groups of different PRs and
317 microalgae species [2]. In the present study, the concentration of erythromycin and
318 clarithromycin at the microalgae cell walls was analysed after the incubation period and
319 we did not find any trace of these antibiotics. This indicates that microalgae
320 bioadsorption was not the cause of erythromycin and clarithromycin removals.
321 Microalgae can also take up organic pollutants along with growth nutrients through
322 bioaccumulation [2]. In this study, microalgae cells were disrupted and analysed; no
323 erythromycin or clarithromycin was observed in the cell biomass, indicating that
324 microalgae bioaccumulation was not the cause of these antibiotics' removal either.
325 By process of elimination, it therefore, seems that biodegradation by microalgae was the
326 means of erythromycin and clarithromycin removal in these experiments.
327 This conclusion is supported by the fact that in samples with a very high concentration
328 of PRs, CHigh, where the microalgae suffered a severe inhibition, the concentrations of
329 the two antibiotics were similar in samples with and without microalgae (Figure 2).
330 In summary, it can be concluded that *Chlamydomonas acidophila* seems to degrade and
331 reduce antibacterial activity of the antibiotics erythromycin and clarithromycin and
332 enhances their removal from wastewater by 93 - 65% and 64 - 50% respectively.

3.2. *Influence of the different pharmaceuticals on growth and nutrient recovery of C. acidophila*

Experimental data on cell growth and nutrients concentration dynamics in SM, WW, WW+Met, CHigh and CModerate with and without microalgae samples during Phase 1 are shown in Fig. 3.

A pH-dependent equilibrium exists between the soluble ammonium ion (NH_4^+) and dissolved molecular ammonia (NH_3). High pH favours ammonia volatilization by driving the equilibrium between NH_3 and NH_4^+ to molecular ammonia [47]. Also, when the pH is high, orthophosphate can be easily removed from wastewater by precipitation as calcium and magnesium salts [48,49]. In the present study, the pH remained below 3 in all samples during the incubation, due to the acidophilic tendency of the microalgae tested, and in samples without microalgae, cells and nutrient concentrations remained constant during the incubation. Therefore, it can be assumed that any decrease in nutrients (N, P) concentration in the rest of the samples was entirely attributed to their assimilation by microalgae.

No significant differences in ammonium and phosphate removal rates were observed between SM samples and WW samples, both achieving values of around $9 \text{ mgNH}_4 \text{ L}^{-1} \text{d}^{-1}$ and $3 \text{ mgPO}_4 \text{ L}^{-1} \text{d}^{-1}$, indicating that the microalgae were not affected by the presence of wastewater. It is acknowledged that light availability is one of the greatest challenges for microalgae cultivation and a high photosynthetic efficiency is essential to decrease the costs of microalgal biomass production. Based on the results obtained in these experiments, it seems that at this low light intensity ($43 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), *C. acidophila*'s growth rate was higher than other species used for wastewater treatment. Furthermore, the ammonium removal efficiencies were similar to those reported in studies with other microalgae in which ammonia volatilization and phosphate

precipitation was controlled by pH [50,51]. The phosphate recoveries were slightly higher than reported by Franchino et al. [51] using *Neochloris oleabundans*, *Chlorella vulgaris* and *Scenedesmus obliquus*, who achieved up to 1.13, 1.96 and 1.50 mg PO₄ L⁻¹ d⁻¹ respectively, and Aslan and Kapdan [50] using *Chlorella vulgaris*, who achieved 1.8 mg PO₄ L⁻¹ d⁻¹. Therefore, *C. acidophila* has shown promise as an agent to recover nutrients under very low light intensities.

FIGURE 3

Regarding the effect of PR on growth, CModerate showed a typical batch growth with an exponential increase of the cell concentration similar to the samples in standard growing media (SM) and wastewater without PRs (WW) (Figure 3). Furthermore, no significant differences were observed in nutrient removal rates when comparing CModerate, SM and WW. This means that *C. acidophila* was not affected by PR concentrations greatly exceeding environmentally relevant concentrations high levels reported in the literature.

However, at the highest PR concentration (CHigh) cell growth was highly suppressed and nutrients removal non-existent, which is consistent with pronounced inhibition. This inhibition could be caused by a high concentration of pharmaceuticals such as clarithromycin and erythromycin; Half Maximal Effective Concentration (EC50) values of 12 - 46 µg/L and 20 – 366 µg/L and No Observed Effect Concentrations (NOEC) values of <40 µg/L and 10 µg/L, respectively, are reported for green algae [52–56], so that the antibiotics, alone or in association with other pharmaceuticals, could have an effect on the microalgae. Furthermore, the high concentration of methanol added in the dosing of these high concentration of pharmaceuticals might also had an effect on this inhibition, as the cell growth and nutrients removals in samples WW+Met were slightly different to the samples where the microalgae did not suffer any inhibition (Figure 3).

It is important to point out that the high concentrations added in CHigh samples were selected in order to stress the system to the limit (at 1,000-fold the highest concentrations reported in literature) and therefore are unlikely to be found in any wastewater treatment plant.

Experimental data on cell growth and nutrients concentration dynamics in SM, WW environmentally relevant concentrations that can be found in any WWTP (CEnv) with and without microalgae samples (Phase 2) are shown in Fig. 4.

FIGURE 4

CEnv showed similar growth and nutrients removals to the samples in standard growing media (SM) and wastewater without PRs (WW) (Figure 4). Furthermore, no significant differences were observed when comparing these data with CModerate (Figure 3). Therefore, based on the obtained results, it can be stated that *C. acidophila* does not seem to be affected by the presence of these 8 pharmaceuticals in wastewater at the maximum concentrations detected in urban effluents. Given that the PR concentration margin for when the microalgae started to show signs of inhibition is very wide (higher than the concentration added in samples CModerate), it seems that *C. acidophila* is very resistant to these micropollutants and therefore suitable for application in wastewater treatment, even though long term exposure experiments should be carried out to verify the chronic resistance ability of this species.

4. Conclusions

Chlamydomonas acidophila seems resistant to the studied pharmaceuticals and to be able to degrade the antibiotics erythromycin and clarithromycin, two of the three macrolide antibiotics in the Watch List, better than other species. Reducing these compounds from wastewater, the antimicrobial selection pressure decreases, which is the on-going continual exposure to low levels of antibiotics and is the primary driver for

AMR development and maintenance. Furthermore, this microalga exhibited a high assimilation of ammonium and phosphates reaching values of around 9 mgNH₄ L⁻¹d⁻¹ and 3 mgPO₄ L⁻¹d⁻¹ compared to other species and it does not seem to be affected by the presence of atenolol, caffeine, carbamazepine, clarithromycin, erythromycin, lidocaine, propranolol and simvastatin in wastewater at concentrations well above those detected in urban effluents. Therefore, *Chlamydomonas acidophila* appears to be a promising agent for wastewater treatment, also contributing to the achievement of phosphate and ammonium standards pertaining to both discharge consents and WFD status assessments, and mitigate eutrophication issues in receiving waters. Further long term exposure experiments should be carried out to verify the chronic resistance ability and antibiotics' removals of this species.

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The authors of this manuscript have no conflict of interest to declare.

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Figure captions

Fig 1. Changes in concentrations of caffeine, simvastatin, lidocaine, carbamazepine, atenolol and propranolol in the medium during the incubation in CModerate samples. The changes in concentrations in CEnv and CHigh followed similar patterns (data not shown). Each point represents the mean value from three replicate determinations with standard deviation.

Fig 2. Removals of erythromycin and clarithromycin in the medium during incubation, expressed as the ratio between their concentrations in the media (C_x) and their initial concentration (C_0). Each point represents mean value from three replicate determinations with standard deviation.

Fig 3. Phase 1: Changes in cells, NH_4^+ and PO_4^{-3} concentration in the medium during the incubation of SM, WW, WW+Met, CHigh and CModerate with and without microalgae. Each point represents mean value from three replicate determinations with standard deviation.

Fig 4. Phase 2: Changes in cells, NH_4^+ and PO_4^{-3} concentration in the medium during the incubation of SM, WW and CEnv with and without microalgae. Each point represents mean value from three replicate determinations with standard deviation.

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Table 1. Pharmaceutical concentrations studied in this assay.

Compound	Reported wastewater Concentrations (µg/L)	Phase 1		Phase 2	References
		CHigh ^a (µg/L)	CModerate ^b (µg/L)	CEnv ^c (µg/L)	
Atenolol	0.35-1.710	6,030	350	6.03	[21]
	3				[22]
	1.35-6.03				[23]
Caffeine	0.68-11.44	54,700	680	54.70	[24]
	22-25.9				[25]
	54.7				[26]
Clarithromycin	0.56	800	40	8.00	[27]
	0.17-0.36				[23]
	0.04-1.2-8				[28]
Carbamazepine	0.113	820	160	0.82	[29]
	0.16-0.820				[21]
	<2.15				[24]
	0.16-0.57				[23]
Erythromycin	0.2	2,300	14	2.30	[22]
	0.11				[23]
	0.014-0.092-0.6				[28]
	1.2				[30]
	2.3				[31]
Lidocaine	0.66-0.77	770	70	0.77	[23]
	0.13-0.18				[21]
	0.07-0.27				[32]
Propranolol	0.03-0.07	510	30	0.51	[23]
	0.001-0.017				[33]
	0.036-0.51				[34]
Simvastatin	0.9	8,500	900	8.50	[22]
	1.23				[35]
	n.d.-2.65-8.5				[8]

(a) **CHigh:** Highest Reported Concentration x1000 (µg/L)(b) **CModerate:** Lowest Reported Concentration x1000 (µg/L)(c) **CEnv:** Highest Reported Concentration (µg/L)

SUPPLEMENTARY INFORMATION

Ionisation mode and transitions for quantitation of pharmaceuticals by high resolution mass spectrometry (HRMS).

Pharmaceutical	Ionisation mode	Precursor Ion	Product Ion
Atenolol	Positive	267.17	190.0868
Caffeine	Positive	195.09	138.0663
Clarithromycin	Positive	748.48	158.118
Carbamazepine	Positive	237.1	194.0972
Erythromycin	Positive	734.47	158.1176
Lidocaine	Positive	235.18	86.0967
Propranolol	Positive	260.16	116.1071
Simvastatin	Negative	419.2792	NA