The effect of CO_2 addition and hydraulic retention time on pathogens removal in HRAPs

Graziele Ruas, Sarah Lacerda Farias, Priscila G. Scarcelli, Mayara L. Serejo and Marc A. Boncz

ABSTRACT

The influence of CO_2 addition and hydraulic retention time (5 and 7 days) on removal of *Pseudomonas aeruginosa, Clostridium perfringens, Staphylococcus* sp., *Enterococcus* sp., and *Escherichia coli* was evaluated in a system with three parallel 21 L high rate algal ponds. Both the addition of CO_2 and an increase in HRT had no significant influence on bacterial removal, but bacterial removal was higher than found in previous studies. The removal was 3.4–3.8, 2.5–3.7, 2.6–3.1, 2.2–2.6 and 1.3–1.7 units log for *P. aeruginosa*, *E. coli*, *Enterococcus* sp., *C. perfringens*, and for *Staphylococcus* sp., respectively. Although CO_2 addition did not increase disinfection, it did significantly increase biomass productivity (by ≈60%) and settleability (by ≈350%). Additionally, even at the lower 5-day hydraulic retention time, CO_2 addition improves removal of chemical oxygen demand (COD), total organic carbon (TOC), total organic nitrogen and phosphorus by 97, 91, 12 and 50%, respectively.

Key words | *Clostridium perfringens*, disinfection, domestic wastewater, sanitation, scrubbing, *Staphylococcus* sp

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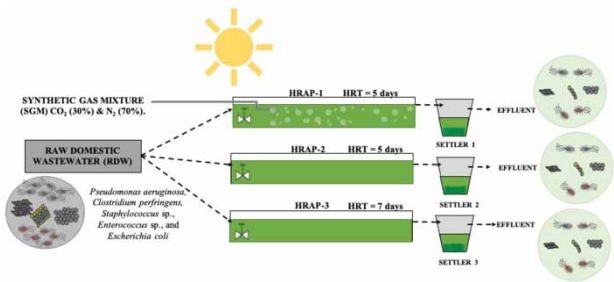
HIGHLIGHTS

- Pathogens removal was not affected neither by CO_2 addition nor by HRT.
- The removal of *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Enterococcus* sp. and *Escherichia coli* was higher than reported in previous studies.
- Removal efficiency of up to 3.8 units log was obtained for *Pseudomonas aeruginosa*.
- CO_2 addition enhanced biomass productivity and settleability.
- Higher HRT increased carbon and nutrients removal.

GRAPHICAL ABSTRACT



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INTRODUCTION

Pathogenic microorganisms and inadequate sanitation are responsible for the spread of numerous diseases and bacterial infections in the world (WHO 2018). The main route of entry of these microorganisms into the aquatic environment is through direct discharge of untreated or inadequately treated wastewater. Despite increasing coverage of basic sanitation services for the world's population, there is still significant inequality, especially in developing countries, and in 2017, 673 million people were still practicing open defecation, facilitating the dissemination of waterborne diseases (UNICEF & WHO 2019). Even where sewage is collected, it is often discharged into water bodies without treatment, as is the case, for instance, with 44% of sewage in Brazil and 78% of sewage in India. Furthermore, the intensive use of antibiotics, and their release into the environment, has accelerated the development of antibiotic resistant bacteria (ARBs) and antibiotic resistant genes (ARGs) (Bouki *et al.* 2013). These multiresistant organisms increase the risks to human and animal health because they make it difficult to control infections (Rizzo *et al.* 2013).

Considering that pathogens are always present in the natural aquatic environment, even though often only in small amounts, and that detection is not always simple to realize, usually indicator organisms, which are present in faecal material and with characteristics that may be related to pathogens in general, are used to detect contamination. *Escherichia coli* is considered the universal indicator due to its high prevalence, and is related to various gastrointestinal infections that may even result in chronic sequelae (Aw 2018). Other common inhabitants of the intestinal tract of warm-blooded organisms, and used as indicator organisms, are *Enterococcus faecalis* species, which have the aquatic environment as their natural habitat but are very resistant, making their detection related to fecal contamination (Motlagh & Yang 2019). Other sanitary-relevant bacteria may also be used as alternative indicators in monitoring, like for instance *Pseudomonas, Clostridium* and *Staphylococcus* (Coronel-Olivares *et al.* 2011). These bacteria are nowadays frequently resistant to antibiotics and, when resistant, can cause serious infections that were considered 'under control', such as dysentery (Motlagh & Yang 2019). The conventional technologies used in wastewater treatment plants (WWTPs) are not efficient in removing pathogenic bacteria, and in fact WWTPs are hotspots for the propagation of multi-resistant organisms, as in these plants optimal conditions for the appearance of new, resistant variants are present due to high bacterial density, availability of nutrients, and the possibility for gene exchanges (Bouki *et al.* 2013; Rizzo *et al.* 2013).

Microalgal-bacterial systems, used in process units such as high rate algal ponds (HRAPs), constitute a sustainable, low-cost and efficient alternative for the treatment of effluents (Posadas *et al.* 2015). Recently, satisfactory results were shown using HRAPs for the inactivation of pathogenic bacteria (Ruas *et al.* 2018). The operating conditions of such reactors, such as HRT, pH and concentration of CO_2, can be changed according to the pollutants present and the required removal rates. For example, in the majority of domestic wastewaters, carbon is limiting, and thus the addition of CO_2, decreasing this limitation, can improve the removal of organic matter and of nutrients, resulting in increased productivity (Park & Craggs 2010). In this case, the necessary CO_2 can be easily obtained from a preceding anaerobic treatment of the wastewater, or from anaerobic digestion of excess sludge at the WWTP itself, or even from fossil fuel combustion for power generation, if a power plant is located nearby.

However, there are still few studies evaluating the pathogen removal performance of HRAPs, and understanding the removal mechanisms and the effects of operating conditions on this removal is critical for determining the best configuration for these systems, in which nutrient removal can then be combined with disinfection and biomass production in a single optimal configuration. Ruas *et al.* (2018) investigated the influence of CO_2 addition on pathogen removal in an HRAP. However, in that study only a single HRAP was operated, and thus the different stages were characterized by different environmental conditions. Simultaneous operation of at least two reactors would greatly improve the comparative analysis of the impact of CO_2 addition and other parameters on pathogen removal. Thus, the aim of this work was to study, in parallel reactors, the influence of CO_2 addition and hydraulic retention time (HRT) on the removal of *Pseudomonas aeruginosa, C. perfringens, Staphylococcus* sp., *Enterococcus* sp. and *E. coli* from raw domestic wastewater (RDW).

MATERIALS AND METHODS

Experimental setup and operational conditions

The experimental setup consisted of three 21 L pilot HRAPs with 0.13 m^2 surface area and 16 cm cultivation broth depth

(called HRAP-1, HRAP-2 and HRAP-3). A liquid recirculation velocity of 20 cm s^{-1} was maintained in each reactor, by means of a submerged pump with a nominal flow rate of 650 L h^{-1} (Sarlo Better B650, Brazil). The effluent of each reactor passed through a 1 L settler with a hydraulic retention time (HRT) of 6 h for HRAP-1 and HRAP-2, and 8.4 h for HRAP-3 (Figure 1).

Microorganisms and culture conditions

The reactors were inoculated with 1.3 L microalgae culture (≈97% *Chlorella vulgaris*) with 0.9 g L^{-1} of total suspended solids (TSS) and with 0.28 L of a nitrifying-denitrifying active sludge (4.7 gTSS L^{-1}) from a WWTP in Campo Grande-MS, Brazil. Microalgae were collected and acclimated to the RDW prior to inoculation of the HRAPs, according to Serejo *et al.* (2015).

Raw domestic wastewater and synthetic gas mixture

RDW was collected once a week from a WWTP in Campo Grande-MS (Brazil) and stored in a 300 L agitation cooling tank (Implemis, Brazil) at 4 °C, in order to prevent sedimentation of suspended solids and degradation of organic matter. The initial concentrations of soluble chemical oxygen demand (COD), total organic carbon (TOC), inorganic carbon (IC), total organic nitrogen (TN), ammonium ion ($N\text{-}NH_4^+$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), phosphorus (P), and non-soluble *P. aeruginosa*, *C. perfringens*, *Staphylococcus*, *Enterococcus* sp. and *E. coli*, as well as pH and TSS of the RDW, are summarized in Table 1. A synthetic gas mixture (SGM) composed of CO_2 (30%) and N_2 (70%) (White Martins, Brazil) was used as source of CO_2 for the pathogen removal efficiency experiments in HRAP-1, passing 2.5 ± 0.4 mL CO_2 min^{-1}. In the HRAP-2 and in the HRAP-3, no CO_2 was added.

Continuous experiments and sampling

The three HRAPs were continuously fed with RDW using peristaltic pumps (Watson Marlon 505 U, UK) applying an HRT of 5 days in HRAP-1 and HRAP-2 and an HRT of 7 days in HRAP-3, and with (HRAP-1) or without (HRAP-2, HRAP-3) using a synthetic gas mixture, in order to study the influence of CO_2 dosing and HRT on the removal efficiency of *P. aeruginosa*, *C. perfringens*, *Staphylococcus*, *Enterococcus* sp. and *E. coli*. During the experiment, three times a week, at 10:00 a.m., 300 mL liquid samples

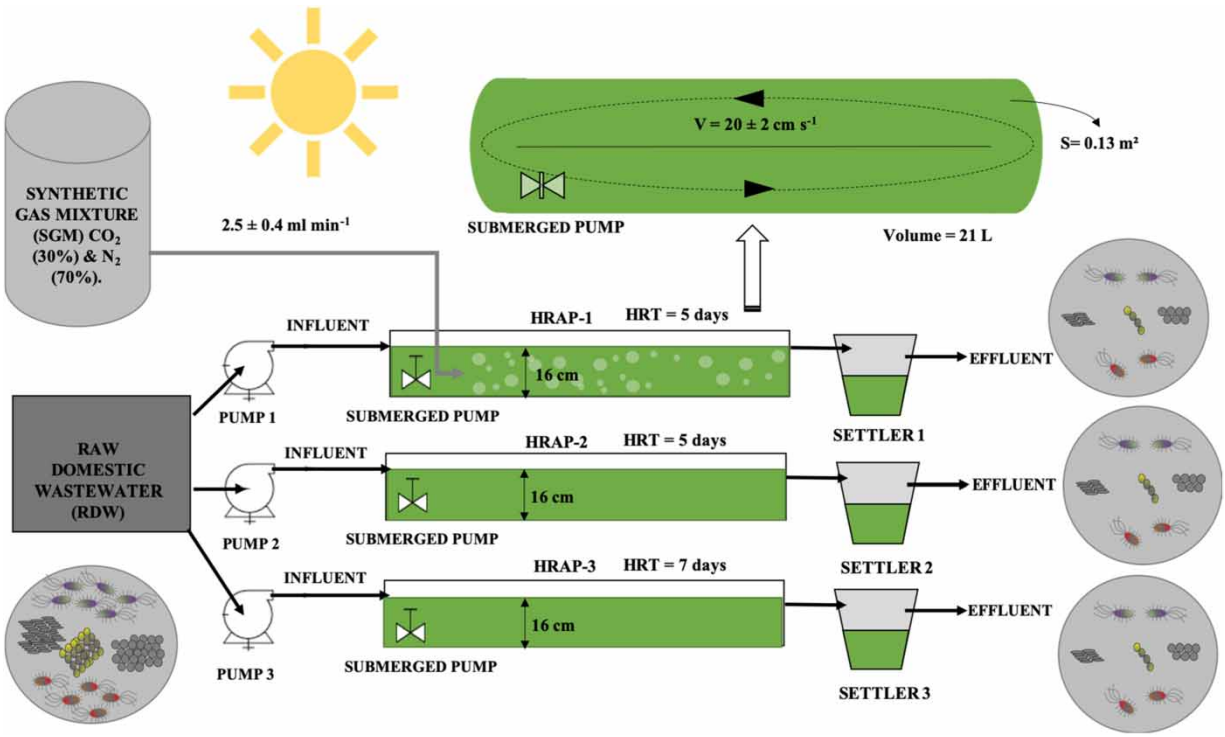

Figure 1 | Experimental setup of three HRAPs (20 L) for pathogen and nutrient removal from RDW.

Table 1 | Initial physical-chemical and biological characterization of RDW

Parameter	Unit	Average ± st.dev.
COD	mg COD L^{-1}	79.5 ± 19.5
TOC	mg C L^{-1}	133 ± 15.3
IC	mg C L^{-1}	56.7 ± 10.5
TN	mg N L^{-1}	122.3 ± 32.9
NH_4^+	mg N-NH_4^+ L^{-1}	37.2 ± 21.7
NO_2^-	mg N-NO_2^- L^{-1}	0.0 ± 0.0
NO_3^-	mg N-NO_3^- L^{-1}	0.2 ± 0.2
P	mg P-PO_4^{3-} L^{-1}	5.5 ± 1.4
C:N	–	1.2
C:N:P	–	34.5/29/1
pH	–	8.3 ± 0.6
Pseudomonas aeruginosa	UFC (100 mL) $^{-1}$	$(6.8 \pm 6.6) \times 10^4$
Clostridium perfringens	UFC (100 mL) $^{-1}$	$(3.4 \pm 2.5) \times 10^5$
Staphylococcus sp.	UFC (100 mL) $^{-1}$	$(1.7 \pm 1.9) \times 10^7$
Enterococcus sp.	UFC (100 mL) $^{-1}$	$(4.1 \pm 4.9) \times 10^5$
Escherichia coli	MPN (100 mL) $^{-1}$	$(7.9 \pm 1.2) \times 10^5$

MPN, most probable number; *CFU*, colony forming units.

were withdrawn from the influent and the effluent (settler output, Figure 1) to determine the concentrations of pathogens, TSS, and (filtration through 0.45 μm glass fibre filters prior to analysis) soluble COD, TOC, IC, TN, N-NH_4^+, NO_2^-, NO_3^- and P. Also, 100 mL liquid samples were withdrawn from the HRAPs to determine the concentrations of pathogens and TSS in the cultivation broth. Light intensity, pH, dissolved oxygen (DO), and ambient and cultivation broth temperatures were monitored daily. The evaporation rate was measured from the difference between measured influent and effluent flows (Serejo *et al.* 2015). The experiment was conducted at the Effluents Laboratory of the Federal University of Mato Grosso do Sul (UFMS) in Campo Grande-MS (Brazil), for 40 days, at a temperature of ≈26 °C.

Analytical procedures

The TOC, IC and TN were determined using a total organic carbon analyzer (Vario TOC Cube, Elementar, Germany). N-NH_4^+ was measured using an Orion Five Star multiparameter meter (Thermo Scientific, The Netherlands), while the pH was measured with a Hanna pH meter HI2211 (Hanna Instruments, Brazil). DO and temperature in the HRAPs were measured using a Jenway 9500 DO2 Oximeter (Jenway, UK). NO_2^-, NO_3^-, and P-PO_4^{3-} were analyzed using a Dionex UltiMate ICS 1100 ion chromatography system with an IonPac AG19/AS19 column (Thermo Scientific, USA). *P. aeruginosa*, *C. perfringens*, *Staphylococcus* sp., *Enterococcus* sp. were determined using the membrane filtration method with M-PA Agar Base – M1121, M-CP Agar Base M1354, Baird Parker Agar Base M043 (HIMEDIA, India) and Agar Base M-Enterococcus K25-610134 (KASVI, Brazil), respectively. *E. coli* was determined using Colilert® kits (IDEXX Laboratories, USA). The light intensity (PAR – Photosynthetically Active Radiation) was recorded using a Quantum meter MQ-200 (Apogee Instruments, USA). The identification of microalgae was conducted by microscopic examination (Olympus BX41, USA) of samples fixed with 5% Lugol's solution and stored at 4 °C prior to analysis, according to Sournia (1978).

Statistical treatment

The results were evaluated using an analysis of variance (ANOVA) with a Fisher's least significant difference (LSD) test applying a 95% confidence level, using Microsoft Excel.

RESULTS AND DISCUSSION

Operational and environmental conditions

The C:N ratio of the RDW was 1.2 (Table 1), lower than the ≈3:0 C:N ratio generally found in RDW (Posadas *et al.* 2015), and far below the ideal C:N ratio for the growth of microalgal biomass of C:N ≈5.6 (Borowitzka & Borowitzka 1988), suggesting a carbon limitation during the treatment. The habits and lifestyle of the population directly influence the wastewater composition and consequently the C:N. The tropical climate and unlimited access to water may have influenced the low C:N ratio found. When there is carbon limitation (a low C:N ratio), the biomass growth rate is low, and a relatively high pH may be reached. Consequently, the removal of nutrients may be incomplete, since at higher pHs (>9) ammonia removal occurs by volatilization and phosphorus removal by precipitation. In addition, at this pH, inhibition of microalgal and bacterial growth may occur due to the presence of free ammonia. That is why numerous studies have endeavored to increase the C:N ratio, present in domestic wastewater, through the direct addition of CO_2 (Park & Craggs 2010).

During the 40 days of operation, the temperatures of the cultivation broths inside the three HRAPs were similar

(≈22 °C), and within the range of between 20 and 30 °C considered optimal for the growth of most microalgal species. At the same time, mean ambient temperature was 25.6 ± 4.7 °C and light intensity was 725 ± 157 μmol m^{-2} s^{-1}. Evaporation rates were similar in HRAP-1, HRAP-2 and HRAP-3, at 0.9 ± 0.7, 0.7 ± 0.7 and 0.8 ± 0.7 L m^{-2} d^{-1}, respectively (Table 2), somewhat lower than the evaporation rates of ≈1.3 L m^{-2} d^{-1} reported by Guieysse for HRAPs under outdoor conditions (Guieysse *et al.* 2013).

The pH and DO in HRAP-2 were 8.7 ± 0.3 and 8.5 ± 1.4 mg O_2 L^{-1}, respectively. Higher results were recorded in HRAP-3 (9.5 ± 0.2 and 9.2 ± 1.6 mg O_2 L^{-1}). This higher pH in HRAP-3 was attributed to the higher HRT (7 days) in combination with the low buffer capacity of the RDW, due to low IC concentrations (Posadas *et al.* 2015). On the other hand, the pH and DO in HRAP-1 of 7.8 ± 0.8 and 7.8 ± 1.3 mg O_2 L^{-1}, respectively, were lower than those found in HRAP-2. The CO_2 addition in the HRAP-1 thus showed to promote an acidification of the cultivation broth, which in its turn led to better conditions for nitrifying–denitrifying sludge (Park & Craggs 2010). In all reactors, DO was above 2.0 mg O_2 L^{-1}, thus aerobic conditions were maintained (Posadas *et al.* 2014a).

Carbon and nutrient removal efficiencies

The removal efficiencies of COD and TOC were higher in HRAP-3 (67 ± 6 and 69 ± 5%, respectively) than in HRAP-1 (41 ± 5 and 45 ± 3%) and in HRAP-2 (34 ± 4 and 36 ± 6%), due to higher bacterial activity at higher HRT, as also observed by Posadas *et al.* (2014b). Likewise, IC removal from HRAP-3 (80 ± 3%) was also higher than from HRAP-1 (67 ± 8%) and HRAP-2 (64 ± 2%), as probably the higher HRT permitted higher C stripping from HRAP-3 (Posadas *et al.* 2014b). The average removal of TN and TP in HRAP-3 were 90 ± 1% and 87 ± 7%, respectively, higher than removals as recorded in HRAP-1 and HRAP-2, where removals of ≈79% and 58% were recorded for TN and TP, respectively (Table 3).

Productivity, settleability and microalgae population

The highest biomass productivity, 3.2 ± 0.2 g m^{-2} d^{-1}, was recorded in HRAP-1, where CO_2 was added, followed by HRAP-3 (2.6 ± 0.2 g m^{-2} d^{-1}) and HRAP-2 (2.0 ± 0.2 g m^{-2} d^{-1}) (Table 3). The addition of CO_2 improved productivity by reducing the carbon deficiency in the RDW, increasing the C:N ratio (Posadas *et al.* 2015; Ruas *et al.*

Table 2 | Results of pH, DO concentration, cultivation broth temperature and evaporation rate obtained during the operation of the three HRAPs

Parameters	Unit	HRAP − 1	HRAP − 2	HRAP − 3
pH	–	7.8 ± 0.8[a] 6.3–10.7[b]	8.7 ± 0.3[a] 6.1–11.0[b]	9.5 ± 0.2[a] 8.2–11.4[b]
DO	mg O_2 L^{-1}	7.8 ± 1.3[a] 4.0–15.7[b]	8.5 ± 1.4[a] 3.7–15.5[b]	9.2 ± 1.6[a] 3.7–16.5[b]
Temperature	°C	21.9 ± 2.7[a] 12.3–28.1[b]	22.3 ± 2.7[a] 12.2–28.3[b]	22.3 ± 2.7[a] 13.1–28.5[b]
Evaporation rate	L m^{-2} d^{-1}	0.9 ± 0.7[a] 0–3.3[b]	0.7 ±0.7[a] 0–3.2[b]	0.8 ± 0.7[a] 0–4.7[b]

[a]Variations and standard deviation.
[b]The minimum–maximum values measured for each parameter.

Table 3 | Removal efficiency (%) of COD, TOC, IC, TC, TN, TP and TSS; TSS concentration and biomass productivity in the three reactors

	Removal efficiency (%)							
HRAP	COD	TOC	IC	TN	TP	TSS (settler)	Productivity (g m^{-2} d^{-1})	TSS (mg L^{-1})
1	41 ± 5	45 ± 3	67 ± 8	78 ± 4	58 ± 9	49 ± 12	3.2 ± 0.2	151.5
2	34 ± 4	36 ± 6	64 ± 2	80 ± 6	58 ± 5	−19 ± 39	2.0 ± 0.2	89.9
3	67 ± 6	69 ± 5	80 ± 3	90 ± 1	87 ± 7	32 ± 11	2.6 ± 0.2	184.5

2018). Biomass productivity was still somewhat lower though than the 5 g m^{-2} d^{-1} obtained by Posadas *et al.* (2014b) in the co-treatment of domestic wastewater with fish farm wastewater in a 180 L outdoor HRAP, with a 7-day HRT and a much higher C:N ratio of ≈100:25. The best settleability, of ≈ 49%, was obtained in HRAP-1. This is in line with the observation by Park & Craggs (2010) that CO_2 addition in HRAPs may promote aggregation/bioflocculation of the microalgae with bacterial flocs, increasing biomass settling. At the beginning of the experiment, the HRAPs were inoculated with a consortium formed mainly by *Chlorella* sp. Gradually, *Scenedesmus* sp., which is commonly found in outdoor HRAPs (Posadas *et al.* 2015), replaced the original biomass, obtaining 100% dominance in HRAP-1, 99% in HRAP-2 and 90% in HRAP-3.

Influence of CO_2 and HRT on removal efficiency of pathogens

P. aeruginosa-RE

The removal efficiency in units log (log-RE) of *P. aeruginosa* was statistically similar in the three HRAPs, between 3.4 and 3.8 log-RE (Figure 2), higher than the 1.6 and 2.5 log-RE reported by Ruas *et al.* (2018) when treating domestic wastewater in a 180 L HRAP with and without CO_2 addition, respectively. However, the first operational difference between the two studies was the way of adding CO_2: an absorption column (100 cm) was used in the study of Ruas *et al.* (2018), whereas in the present study the addition was performed by bubbling CO_2 at the bottom of the reactor (16 cm depth). The absorption column provides more contact time and a closed environment, increasing the transfer rate of CO_2 from gaseous to the liquid phase, while bubbling is limited in terms of CO_2 dissolution in the cultivation broth (Serejo *et al.* 2015), and thus had no significant effect on the removal of *P. aeruginosa* in the present study. Additionally, the second operational difference is the light intensity, which in our current study (at ≈725 μmol m^{-2} s^{-1}) was almost double the maximum recorded by Ruas *et al.* (2018). Light intensity (photo-oxidative damage) is one of the main factors in the inactivation of *P. aeruginosa* and other bacteria, which may explain the higher removal efficiency in the present study.

Furthermore, each microalgae produces different toxins, antibiotic substances and photosynthetic pigments, so they can affect pathogenic bacteria differently (Evangelista *et al.* 2008). In the earlier experiments of Ruas *et al.* (2018), different microalgae were present in each stage: *Chlorella* sp. and *Microscopora* sp., in stages I and II, respectively, possibly caused by the CO_2 addition, and the author indicated this as one of the possible causes of different *P. aeruginosa*-RE. Under the current experimental conditions, in spite of the CO_2 addition, the microalgae population remained constant in all HRAPs, which could explain the similar *P. aeruginosa*-RE under all conditions. However, further studies are needed to understand the effect of each microalgae genus on the removal of pathogenic bacteria in HRAPs.

Bahlaoui *et al.* (1997) reported ≈ 1.1 log-RE of *P. aeruginosa* treating raw domestic wastewater at 8 days' HRT in a 16,800 L HRAP, conducting experiments for 2 years, testing different HRTs. The authors concluded that the behaviour of

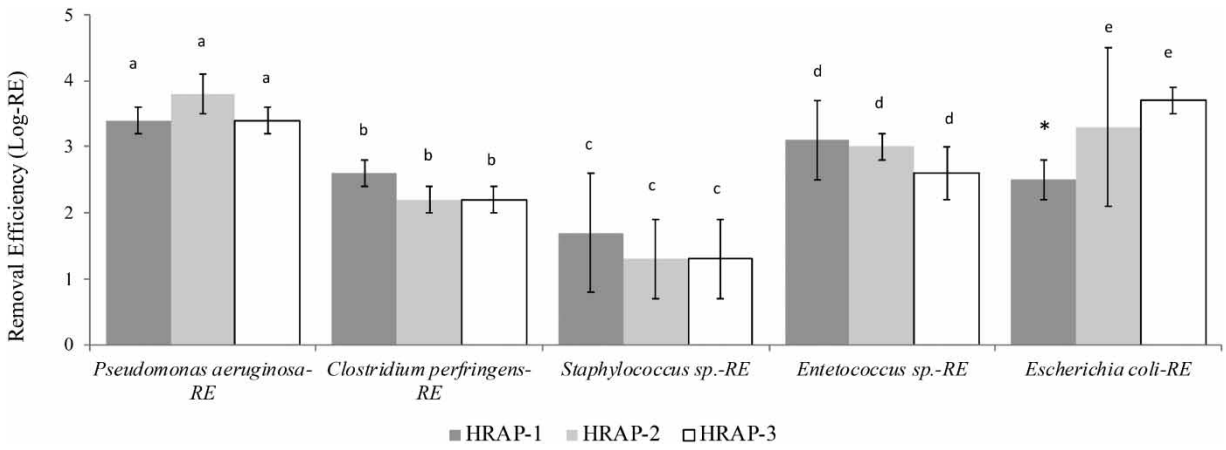

Figure 2 | Average value and standard deviation of removal efficiency in units log of *P. aeruginosa, C. perfringens*, *Staphylococcus* sp., *Enterococcus* sp., and *E. coli*. in HRAP-1 (with CO_2 and HRT ≈5 days), HRAP-2 (HRT ≈5 days) and HRAP-3 (HRT ≈7 days). Means with the same letter are not significantly different ($p < 0.05$) and (*) indicates they are significantly different.

opportunistic pathogen bacteria (*Pseudomonas* sp. and *Aeromonas* sp.) is different from that of faecal-indicator bacteria, and thus the removal mechanisms may be different as well, in terms of acting factors and complexity. The authors also emphasize that light intensity and temperature are important in disinfection in HRAPs, since the best results are obtained in summer, and the HRT might need to be increased in winter as a strategy to improve removal under conditions of lower light intensity and temperature.

C. perfringens-RE

When the treated wastewater is intended for agricultural reuse, *C. perfringens* must be monitored together with *E. coli*, because it is resistant to disinfection due to its ability to form spores when under adverse environmental conditions. Moreover, it is an indicator of enteric viruses and protozoa in treated wastewater (Gutiérrez-Alfaro *et al.* 2018). HRAPs are able to remove *C. perfringens* better than maturation ponds (García *et al.* 2008), because in maturation ponds the exposure to UV radiation and high temperatures is more intense, and this is one of the conditions that start the formation of spores in *C. perfringens* (Raju *et al.* 2007), which makes it difficult to inactivate.

The *C. perfringens*-RE was 2.2 ± 0.2 log-RE in HRAP-2 and in HRAP-3, and 2.6 ± 0.2 log-RE in HRAP-1; these three removal efficiencies were not statistically different (Figure 2). García *et al.* (2008) reported lower *C. perfringens* removal efficiencies in a 461 L HRAP (10 d HRT) and a 15 m^2 maturation pond (20 d HRT) of 1.7 and 1.2 log-RE, respectively, both treating RDW in summer (≈27 °C). When testing two wetland configurations, García *et al.* (2018) obtained higher removals, of 2.63 and 3.42 units log, in a free-water surface wetland and a sub-surface flow wetland, respectively, and UV radiation in the system decreased the *C. perfringens* removal, due to spore formation. On the other hand, Gutiérrez-Alfaro *et al.* (2018) compared three systems for *C. perfringens* removal: HRAP, solar disinfection (SODIS) and dissolved air flotation (DAF), finding lower removal efficiencies, of 0.1, 0.9 and 1.7 log, respectively. In this case, DAF can remove the bacteria even in spore form. Also, the size of the bacteria (diameter ~1 μm and length ~5 μm) facilities the filtration process of this system. However, the conditions of cultivation in the HRAP was not show, it cannot be inferred why the *C. perfringens* removal was so low compared to the present study. Therefore, more studies need to be conducted to determine which mechanisms and parameters act in the *C. perfringens* removal.

Staphylococcus-RE

The addition of CO_2 and the HRT variation had no statistically significant influence on *Staphylococcus*-RE; removal efficiencies of 1.7 ± 0.9, 1.3 ± 0.6 and 1.3 ± 0.6 units log were obtained in the HRAPs 1, 2 and 3, respectively (Figure 2). The removal efficiencies obtained were similar to those found by García *et al.* (2008) with *Staphylococcus* sp.-RE of ≈1.2 log-RE in winter and summer, and observed no statistically significant difference between a HRAP and a maturation pond, both treating the same wastewater. The main parameters affecting *Staphylococcus* sp. removal in synthetic medium and in lab conditions were the luminous intensity and the concentration of biodegradable organic matter (Nola *et al.* 2010), but both the potential and the main mechanisms of *Staphylococcus*-RE in HRAPs are not yet known, and thus more investigation is needed. It is important monitoring *Staphylococcus* sp. in treatment systems, understanding how it can be removed and thus improving the efficiency of the treatment, because this bacterium is pointed out as one of the antibiotic resistance bacteria, and its presence in the environmental can put at risk the health of people and animals (Bouki *et al.* 2013).

Enterococcus-RE

The *Enterococcus*-RE was 3.1 ± 0.6 log-RE in HRAP-1, 3.0 ± 0.2 log-RE in HRAP-2 and 2.6 ± 0.4 log-RE in HRAP-3, statistically similar removal efficiencies (Figure 2). As previously reported by Ruas *et al.* (2018), CO_2 addition does not directly influence removal of *Enterococcus* sp. Similar *Enterococcus* sp. removal (≈2.7 log-RE) was obtained by Ruas *et al.* (2018) in a 180 L HRAP (5 d HRT), while lower RE of ≈ 2.0 and 1.1 unit log was found in a 0.7 m depth HRAP (7 d HRT) and 32 m^2 HRAP (6 d HRT), respectively, by Awuah *et al.* (2002) and Gutiérrez-Alfaro *et al.* (2018). This last work also recorded that *Enterococcus* sp. (mostly *E. faecalis* and *E. faecium*) was more effectively removed by the HRAP than by DAF (0.1 log-RE). According to Awuah *et al.* (2002), the main factor that can act in the *Enterococcus* sp. removal in microalgae systems was light and daily fluctuations of pH and DO.

E. coli-RE

Equal *E. coli* removal efficiencies were found in HRAP-2 and in HRAP-3 (3.3 ± 1.2 and 3.7 ± 0.2 log-RE, respectively), higher than that observed in HRAP-1 (2.5 ± 0.3 log-RE) (Figure 2). *E. coli* are Gram-negative bacteria, being

more susceptible to pH, DO and light variations, when compared to *Enterococcus* sp. (Gram-positive) (Awuah *et al.* 2002). When the pH exceeds a value of 8.5, the kinetic constant of *E. coli* removal increases (Ouali *et al.* 2013). In both HRAPs-2 and -3, favourable conditions for *E. coli* removal were present throughout the experimental period: pH above 8.5 (≈8.7 and 9.5, in HRAP-2 and HRAP-3, respectively), and $>8.5\ mg\ O_2\ L^{-1}$. When the pH is closer to neutral, *E. coli* removal is reduced. For instance, Posadas *et al.* (2015) recorded lower *E. coli* removal of only ≈1.0 log in an 850 L HRAP with a controlled pH (pH ≈7.0) treating domestic wastewater (HRT 2.8 d), while Ruas *et al.* (2018) recorded ≈2.2 log-RE in two stages of operation, with pH of 7.7–6.8. Other parameters affect the *E. coli* removal including DO and light, where a lower intensity of these may have reduced the removal efficiencies.

CONCLUSIONS

The use of 30% CO_2 at $2.5\ mL\ min^{-1}$ in a 21 L HRAP for treatment of raw domestic wastewater with a low C:N ratio resulted in a strong increase in biomass productivity, from 2.0 to $3.2\ g\ m^{-2}\ d^{-1}$, and in improvements in COD and TOC removal rates by 20 and 25%, respectively. On the other hand, the use of a 7-day HRT, when compared to a 5-day HRT, improved the removal of COD, TOC, TN and TP by 97, 91, 12 and 50%, respectively. In general, the removal efficiencies of pathogenic bacteria were similar under the tested conditions, but higher than those reported in previous studies, reaching 3.8, 3.7, 3.1, 2.6 and 1.7 units log for *P. aeruginosa, E. coli*, *Enterococcus* sp., *C. perfringens* and *Staphylococcus* sp., respectively.

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