

Significance of Algae in eliminating and deactivating pathological index Organisms in Wastewater Stabilization Pond Systems

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Abstract. Wastewater stabilization ponds (WSPs) are an economical alternative to conventional wastewater treatment technologies. They are commonly favored by small and rural communities. Due to long hydraulic retention time required by the treatment in these systems, algal activity is often expected during summer season. This study investigated the effects of algal growth on the removal and inactivation of pathogenic indicator organisms in WSP systems. Two predominant algae species (*Mougeotia* sp. and *Nostochopsis* sp) that were found in Iraq (Alkhademiyah sewage station in Baghdad) WSP were able to increase both pH and DO. The highest inactivation rates of both *E. coli* and total coliforms (TC) were observed at pH 10.6 compared to other pH (5.1, 8.2, 8.4, 10.6) investigated in the bench-scale experiments. Both high (20mg/L) and low DO (1 mg/L) levels can facilitate the removal and inactivation of both *E. coli* and TC. Enterococci were significantly reduced at both intermediate (8.6 mg/L) and high (20 mg/L) DO concentrations. Therefore, the presence of algae can potentially promote the removal of *E. coli*, TC and Enterococci. Much higher inactivation of *E. coli*, TC and Enterococci at a higher temperature (20°C) than at a lower temperature (4°C) indicates temperature is one the most important removal factor. Two potential/additional indicator organisms (Enterococci and *Clostridium perfringens* (*C. perfringens*)) exhibited different inactivation trends than the traditional indicators (*E. coli* and TC) under the same pH and DO conditions. *C. perfringens* were tolerant to all the tested pH, DO and temperature conditions. Their resistance to environmental stresses may pose potential health risk. Hence, both Enterococci and *C. perfringens* could be potentially used as indicator organisms to predict the overall level of pathogens in treated wastewater.

1 Introduction

Wastewater stabilization ponds (WSPs) are environmentally sustainable ecosystems that require lower energy inputs and maintenance compared to conventional forms of wastewater treatment [1, 2]. As such, they have become a popular alternative to conventional technologies, especially in small, rural and remote regions. However, the performance of WSPs is highly dependent on environmental and climatic conditions, hence, the treatment efficiency can be sensitive to both location and season.

Maturation WSPs, with depth of 1-1.4 m, are designed for disinfection and are often implemented as a tertiary polishing step in wastewater treatment. Over the past two decades, one of the research interests in WSPs has been to understand the mechanisms of pathogen removal in these systems. Previous studies have shown that the main factors contributing to disinfection in WSP systems are: sunlight, pH, dissolved oxygen (DO), temperature, nutrient limitation, water depth, hydraulic

retention time, attachment, sedimentation and predation [2-8].

As a result of the long hydraulic retention times associated in these systems, algal activity is often expected during warmer seasons. Consequently, high dissolved oxygen (DO) (~ 15 mg/L) and pH levels (~ 9.5) are often observed in WSPs containing algae [1, 3, 8, 9]. It has been noted in the literature that pH and DO are two important factors that can affect the removal and inactivation of pathogenic microorganisms in WSPs [3, 4, 6]. The presence of algae may influence the various treatments occurring in WSP systems, such as nutrient removal and pathogen removal [8]. *Escherichia coli* (*E. coli*), fecal [10, 11] coliforms and total coliforms are common indicator organisms that are used to assess the quality of treated wastewater and determine the presence of pathogenic microorganisms [12, 13]. Their presence can be a good indication of fecal contamination. However, the limitations associated with the use of traditional fecal indicator organisms have been recognized in the literature; particularly as they can sometimes be limited in their

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ability to represent overall pathogen levels [14-16]. A number of potential indicator organisms have been proposed as representative of pathogenic microorganisms in addition to those traditionally employed. Enterococci are one of the potential indicator organisms that have been reported to have better survival rates than fecal coliforms at high pH. In a bench scale study using grab samples of sewage obtained from the Academic Board of Baghdad University treatment plant, [5] observed that Enterococci could survive at pH levels as high as 11. Therefore, the use of *E. coli*, fecal coliforms or total coliforms as indicators may under predict the presence or concentration of Enterococci, especially under high pH conditions. *Clostridium perfringens* (*C. perfringens*), an anaerobic, spore-forming bacteria found in high concentrations in human and animal feces, has been recently used as an additional indicator. Its spore phase is considered highly resistant to environmental conditions, as well as conventional disinfection processes, whereby, [17] found that the inactivation rate of *E. coli* was one order of magnitude greater than that of *C. perfringens*. Therefore, it was suggested that rather than *E. coli*, this microorganism could be used to predict the presence of resistant protozoan and helminthic pathogens such as *Cryptosporidium*, *Giardia* and helminth eggs [18-20]; However, a comprehensive understanding of how these indicator organisms would respond to different pH, DO and temperature conditions is missing in the literature. The main objective of this study was to investigate the indirect effect of algae on the removal and inactivation of traditional (*E. coli* and total coliforms) and alternative indicator organisms (*Enterococcus* and *C. perfringens*). Bench scale experiments were conducted to examine whether the presence of algae could be responsible for the high pH and DO levels in WSP systems, then to assess the effect of the high pH and DO conditions on the removal and inactivation of both typical and potential indicator organisms.

2 Material and Methods:

2.1 Source of wastewater samples Description of the study area

Wastewater samples were collected from Alkhademiya sewage station in Baghdad. extended from southwest of AlKarkh city with their elongation for more than 30 km passes through vast farmlands, orchards and several residential quarters , followed the figure below (Figure .1). The sewage station treats approximately 1,500 m³/day of municipal wastewater. The two WSPs have operating depths of 2.61 m and 4.42 m [1]. Hydraulic retention times (HRTs) were estimated to be 16 and 8 days [2]. Algal samples were collected, while wastewater samples used for bench pH, DO and temperature tests were collected from the secondary effluent, which had been treated through primary treatment, secondary aeration tanks as well as a secondary clarifier. All the experiments in this research paper are well done in the AL Yarmouk University College laboratories in Baghdad.

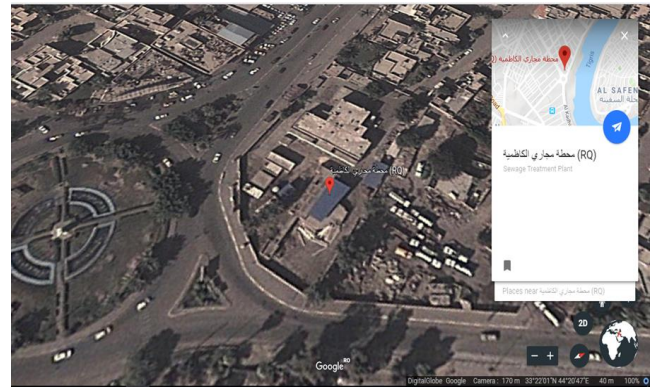


Figure .1. Map of the sampling “Alkhademiya sewage station in Baghdad”.

2.2. Algae cultivation experiments

At the Alkhademiya sewage station in Baghdad , algal blooms are typically observed to start in early June. During the warmer months, from June to September, pH generally ranged from 9.5 to 11 [3]. Two types of algae were found to be predominant . One was *Nostochopsis sp.* , also known as Water Net algae, and tended to float on the water surface. The other one was filamentous algae, *Mougeotia sp.* which preferentially attached to the macrophytes in the WSP.

A Nikon SMZ25 microscope (Nikon Instruments Inc.) was used to take images of algae. Microscopic images of both algae are shown in Figure .2. These two types of algae were collected on August 17, 2016, and transported to the laboratory in a cooler packed with ice. They were washed five times with autoclaved DI water and cultivated in Bold’s Basal Medium (BBM) separately for 6 days at room temperature (~20°C). Three 500-mL Mason jars, each containing 400 mL of BBM and 10 g algae, were prepared for each type of algal. A jar of BBM only (without any algae samples) was also maintained as a control. pH and DO levels were monitored daily using a HQ40d portable multi-parameter (Hach, Loveland, CO).

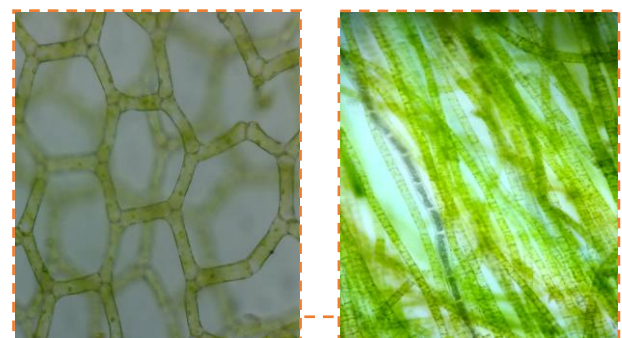


Figure .2. Microscopic images (40X) of two algae, *Nostochopsis sp.* (a) and *Mougeotia sp.* (b), isolated from the Alkhademiya sewage station in Baghdad .

2.3. pH experiments

The purpose of the laboratory pH experiment was to assess the effect of pH alone, on the inactivation of indicator organisms. A grab sample was collected from the treatment plant secondary effluent, which is the influent to the first WSP. Seven Liters of the wastewater grab sample was distributed equally into fifteen 500-mL Mason jars. All Mason jars were autoclaved before use. Five different pH conditions (5.1, 8.2, 8.4, 9.7 and 10.6) were tested in triplicate. pH was adjusted daily with 0.2N HCl and 0.2N NaOH solutions as required. All the jars were placed in an incubator (Thermo Scientific Refrigerated Incubator) with the temperature of 20 °C and with a light intensity of 3400 Lux, measured with a REED LM-81LX Compact Light Meter (Reed instruments), and DO was kept at 8.5 mg/L by bubbling air using aquarium air diffusers. The concentrations of indicator organisms were measured on days 1, 3, 5, 7 and 9.

The indicator organisms selected for this study were *E. coli*, total coliforms, *Enterococci* and *C. perfringens*. Their concentrations were quantified using the membrane filtration technique (Clesceri et al., 1998), and culture medium (Chromocult Coliform Agar (Merk, Germany), Chromocult Enterococci Agar (Merk, Germany), and m-CP Agar base for microbiology (MiliiporeSigma, Canada) (Reinoso et al., 2011; Mosteo et al., 2011; Ouali et al., 2014). Plate counting was performed after a 24-hour incubation period at 37°C for *E. coli*, total coliforms and *Enterococci*, and a 48-hour anaerobic incubation at 44°C for *C. perfringens* in an anaerobic jar (Merk, Germany). In this study, the “inactivation” and “removal” of indicator organisms referred to the disappearance of culturable colonies. The detection limit was 1 CFU/100 mL. If no culturable organisms were found in 100 mL sample, the concentration was noted as zero.

2.4 DO experiments

Three DO levels of 1, 8.5 and 20 mg/L were investigated to simulate low, intermediate (control) and high DO concentrations. pH and temperature were maintained at 7 and 20°C, respectively. A secondary effluent sample was collected from the wastewater treatment plant. Three replicates of each DO condition were prepared. A sample volume of 400 mL was transferred into each of nine 500-mL Mason jars. Low, intermediate and high DO conditions were maintained by constantly bubbling N₂, air and O₂ using aquarium air diffusers, respectively. The concentrations of indicator organisms were measured on days 1, 3, 7, 9, 11 and 13.

2.5 Temperature experiments

The inactivation and removal of indicator organisms were measured at room temperature (20°C) to simulate warmer season, and at an incubator temperature of 4°C to simulate colder season. A grab sample from Alkhademiyah sewage station in Baghdad effluent was obtained. Sample volume of 400 mL was used in each Mason jar. Both temperature condition experiments were conducted in triplicate. pH and DO were kept at 7 and 8.6

mg/L, respectively. The concentrations of indicator organisms were measured on days 1, 3, 5, 8, 10, 13 and 15.

2.6 Statistical analysis

t-test is most commonly applied when the test statistic would follow a normal distribution. The t-test was used to determine if two sets of data are significantly different from each other for different pH, DO and temperature conditions.

3. Results and Discussion

Over a monitoring period of 6 days, pH and DO were noted to increase from 5.2±0.1 to 8.1±0.3, and from 6.1±0.1 to 9.1±0.2, respectively, in *Mougeotia sp.* jars (Figure.3). Similarly, in the *Nostochopsis sp.* jars, pH increased from 6.0±0.1 to 9.0±0.3, and DO from 6.2±0.1 to 7.5±0.4 (Figure .4). In the control Mason jar, pH varied between 5.9 and 6.2, while DO fluctuated between 5.8 and 6.2 (Figure .5). The increases in both pH and DO were considered significant compared to the control ($p < 0.05$). There were significant increases in both pH ($p < 0.05$) and DO ($p < 0.05$) in both *Nostochopsis sp.* and *Mougeotia sp.* jars. This experiment confirmed that the two predominant algal species found were likely responsible for the high pH and DO levels observed.

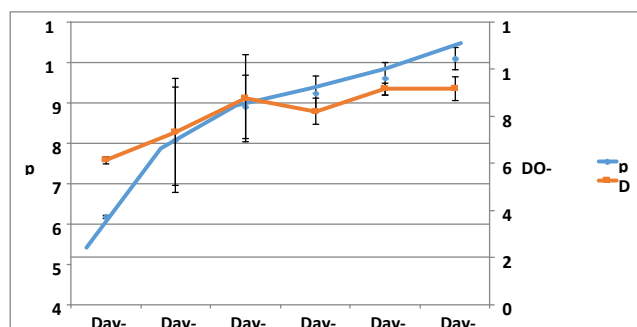


Figure .3. Changes in pH and DO over a 6-day period in the Mason jars containing *Mougeotia sp.* and BBM. Average pH and DO levels presented in this figure were calculated from three replicate jars. Error bars represent standard deviation.

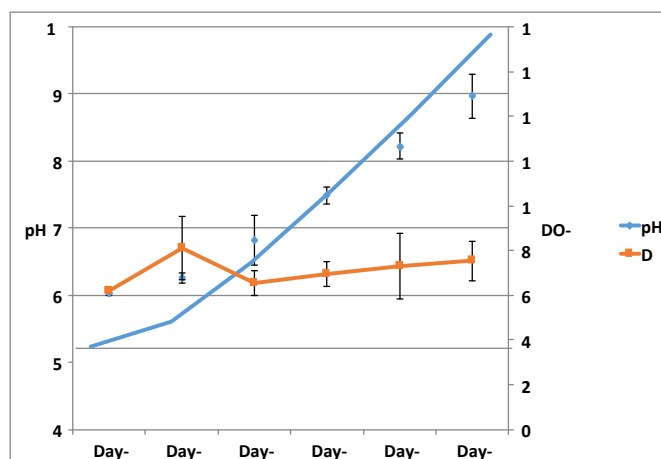


Figure.4. Changes in pH and DO over a 6-day monitoring period in the Mason jars containing BBM and *Nostochopsis sp.* Average pH and DO levels presented in this figure were calculated from three replicate Mason jars. Error bars represent standard deviation.

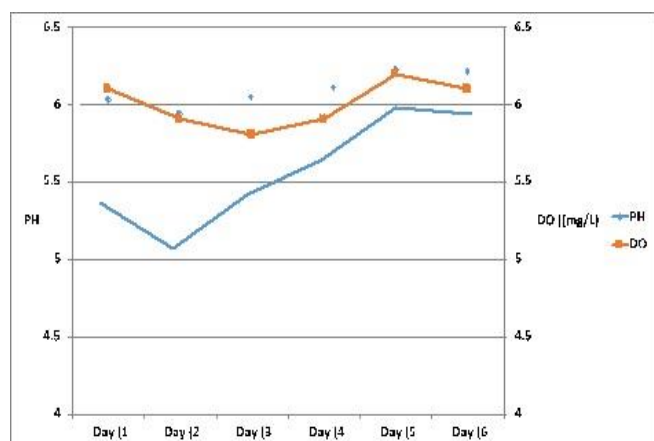


Figure .5. Changes in pH and DO over a monitoring period of 6 days in the control jar containing BBM alone.

The inactivation of four indicator organisms (*E. coli*, total coliforms, *Enterococci* and *C. perfringens*) was tested under five pH conditions, pH 5.1, 9.7, 8.4, 8.2, and 10.6. The initial concentrations of *E. coli*, total coliforms, *Enterococci* and *C. perfringens* in the secondary effluent were 3.16, 4.75, 3.44 and 3.17 log CFU/100 ml, respectively. The original sample with a pH of 8 was used as a control in this study. Inactivation rates of indicator organisms were estimated by the first order rates constants, *k*, which was calculated by using Equation (1) [4, 5] :

$$\ln\left(\frac{C_t}{C_0}\right) = -Kt \quad (1)$$

where C_0 is the initial concentration (CFU/100 mL), and C_t represents the concentration (CFU/100 mL) at time, t . The inactivation of the selected indicator organisms throughout the 9 days of exposure to different pH levels is compared in Figure .6. Table .1 summarized the inactivation rates of the four indicator organisms under the different pH conditions. First order inactivation rate constants for *E. coli* ranged from 0.11 day⁻¹ at pH 8 to 0.19 day⁻¹ at pH 10.5. Inactivation rates for total coliforms varied between 0.08 day⁻¹ at pH 8 and pH 8.4 and 0.12 day⁻¹ at pH 10.5. In general, the inactivation rates of both *E. coli* and total coliforms increased with an increase in pH. However, no significant differences ($p > 0.05$) between the tested pH were found. These observations were consistent with the studies done in the literature. In a study by [6] , it was found that pH of 8 and 8.8 (tested pH include 6.6, 7.3, 8 and 8.8) inhibited the survival of *E. coli*. [7] found that the inactivation rate of *E. coli* increased when pH was above 8.5. A study by [8] and [1] also reported that no significant difference in decay rates of fecal coliforms at pH levels of 5, 7, 9 and 10, and under both sunlight and dark conditions. In a previous study, Liu et al. (2015) reported a strong and

negative correlation between pH and *E. coli* concentrations over a monitoring period of 5 years at the Alkhademiya sewage station - Baghdad and during this monitoring period, *E. coli* concentrations tended to increase when pH decreased below 8. At pH 10.6, it was noted that there was a sharp decay with the inactivation rate of 0.30 d⁻¹ for *E. coli* and 0.23 d⁻¹ for total coliforms during first 4 days.

Table .1. Inactivation rates of four indicator organisms at different pH levels. (DO was at 8.6 mg/L and temperature was at 20°C)

| | <i>E. coli</i> , d-1 | Total coliforms, d-1 | <i>Enterococci</i> , d-1 | <i>C. perfringens</i> , d-1 |
|---------|----------------------|----------------------|--------------------------|-----------------------------|
| pH 8.2 | 0.12 | 0.05 | 0.19 | -0.01 |
| pH 8.4 | 0.13 | 0.07 | 0.21 | -0.01 |
| pH 9.7 | 0.14 | 0.08 | 0.10 | -0.01 |
| pH 10.6 | 0.18 | 0.14 | 0.08 | -0.01 |
| pH 5.1 | 0.16 | 0.12 | * | -0.01 |

*Final concentration was below detection limit of 1 CFU/100mL.

Unlike *E. coli* and total coliforms, a higher removal and inactivation of *Enterococci* was observed at pH 5.1 and pH 8.4. There were no *Enterococci* detected after day 3 at pH 5.1. This observation could challenge the use of *E. coli* and total coliforms to represent pathogenic

Enterococci as these organisms showed different responses to various pH conditions. Inactivation of *E. coli* increased with increasing pH while in contrast, *Enterococci* was noted to exhibit similar rates of inactivation at all pH levels [7-9] also reported that low pH (pH 4 and 5) had a more bactericidal effect on *Enterococci* in domestic wastewater than alkaline conditions. Spore forming bacteria, *C. perfringens*, showed resistance to all the pH levels investigated during the test period. They may require a longer period of time to be inactivated or pH is not a significant removal mechanism for *C. perfringens*.

The effects of different DO concentrations on the removal and inactivation of indicator organisms were also investigated in this study. Table .2 presents the inactivation rates of the four indicator organisms at low, intermediate and high DO levels. Figure .7 shows the concentrations of *E. coli*, total coliforms, *Enterococci* and *C. perfringens* at different DO levels over the 13-day testing period. The highest inactivation rates for both *E. coli* and total coliforms were observed at the high DO concentration of 20 mg/L (Figure .7 (a) and (b)). Among the selected indicator organisms, *E. coli*, total coliforms and *Enterococci* are facultative anaerobes, while *C. perfringens* are obligate anaerobes. Facultative anaerobes can metabolize either aerobically or anaerobically; however these organisms prefer aerobic conditions as aerobic respiration generates more adenosine triphosphate (ATP) than either fermentation or anaerobic respiration. This could explain why comparatively low inactivation rates were generally found under

intermediate DO condition of 8.5 mg/L for both *E. coli* and total coliforms, while high DO concentrations could promote the removal of these two microorganisms. However, no significant differences ($p>0.05$) between the DO concentrations were found. David-Colley et al. (1995) also observed a synergistic increase in inactivation of *E. coli* when both DO and pH increased. The light inactivation of *E. coli* and *Enterococci* have been reported to increase with increasing DO concentrations [7, 8, 10].

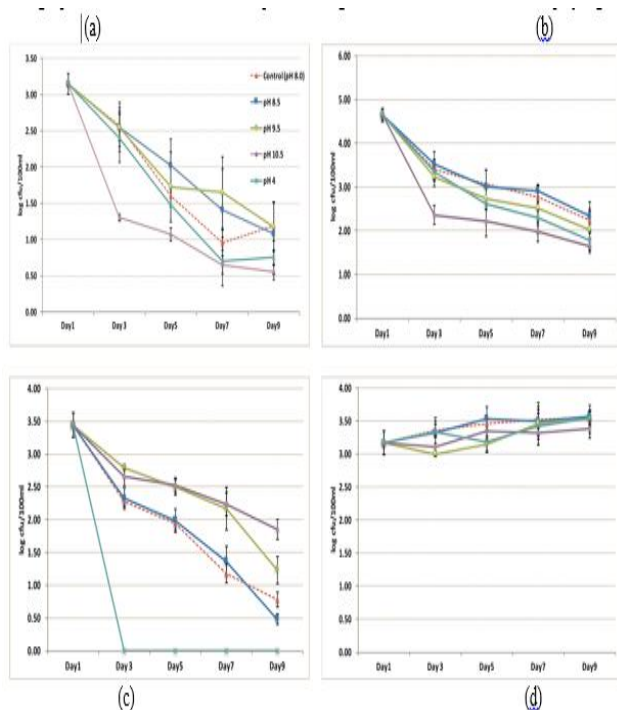


Figure 6. Inactivation and removal of *E. coli* (a), total coliforms (b), *Enterococci* (c) and *C. perfringens* (d) in the secondary effluent wastewater collected from the Alkhademiyah sewage station in Baghdad governorate, over a 9-day period during exposure to pH levels of 5.1, 8.4, 8.2, 9.7 and 10.6.

For the removal and inactivation of *Enterococci*, a higher inactivation rate was found at high and intermediate DO concentrations. The *Enterococci* species obtained from the secondary effluent appeared to exhibit a higher growth rate at lower DO concentrations. Moreover, a significant difference was found between low DO and high DO ($p=0.004$). As such, this would suggest that when DO was enhanced by algal photosynthesis during warm season, it could facilitate the inactivation of *Enterococci*. Although *C. perfringens* are obligate anaerobes, and oxygen can be considered toxic to them, they showed a minimum inactivation rate of 0.01 day^{-1} at high DO concentration over the monitoring period of 13 days. This again shows the resistance to environmental stresses of *C. perfringens*.

Table.2. Inactivation rates of four indicator organisms at low (1 mg/L), intermediate (8.6 mg/L) and high DO (20 mg/L) levels (pH was at 7 and temperature was at 20°C).

| | <i>E. coli</i> , Total day^{-1} | coliforms, day^{-1} | <i>Enterococci</i> , day^{-1} | <i>C. perfringens</i> , day^{-1} |
|--------------------|--|------------------------------|--|---|
| Control (8.6 mg/L) | 0.05 | 0.02 | * | -0.01 |
| High DO (20 mg/L) | * | * | * | 0.01 |
| Low DO (1 mg/L) | * | * | 0.01 | 0.00 |

*Final concentration was below detection limit of 1 CFU/100mL.

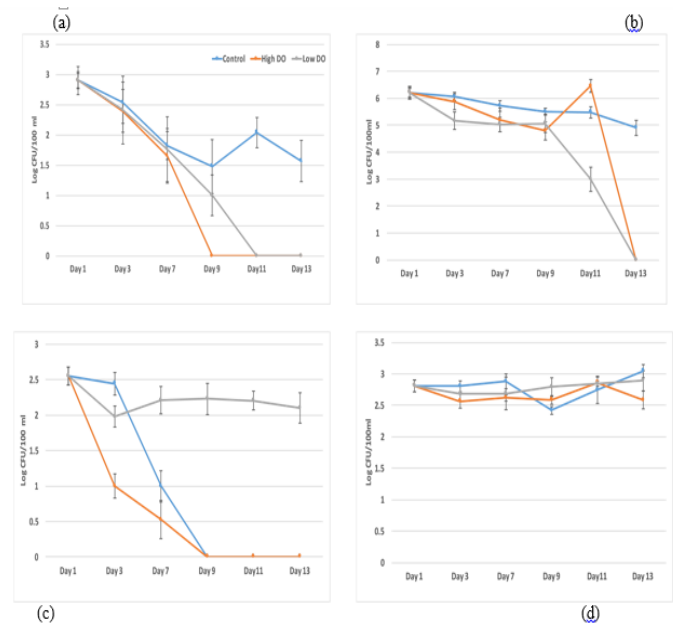


Figure 7. Inactivation and removal of *E. coli* (a), total coliforms (b), *Enterococci* (c) and *C. perfringens* (d) in the secondary effluent wastewater collected from the Alkhademiyah sewage station in Baghdad governorate over a 13-day period when they were exposed to low, intermediate and high DO levels. Temperature were maintained at 20 °C.

Two temperatures (4°C and 20°C) were selected to simulate the cold and warm seasons, and to assess their effects on the removal and inactivation of indicator organisms. Figure 8 shows the concentrations of *E. coli* (a), total coliforms (b), *Enterococci* (c) and *C. perfringens* (d) under warm ($T=20^\circ\text{C}$) and cold ($T=4^\circ\text{C}$) temperatures over a monitoring period of 15 days. The inactivation rates of *E. coli* were -0.02 day^{-1} at 4°C and 0.11 day^{-1} at 20°C, while for total coliforms, the inactivation rates ranged from -0.01 day^{-1} and 0.03 day^{-1} , respectively. There was no *Enterococci* colony detected on day 12 under the 20°C condition and the inactivation rate for the first 10 days was 0.23 day^{-1} . Meanwhile, the inactivation rate of *Enterococci* at 4°C was 0.05 day^{-1} . The concentrations of *E. coli*, total coliforms and *Enterococci* were generally significantly reduced at 20°C compared to at 4°C ($p<0.05$). This finding agrees with observation made by several researchers as they have reported increased removal efficiencies of fecal coliforms

at higher temperatures [26]; Pearson [10, 25, 27]. In a study by [24], fecal coliforms were reduced by 98.95% during the warmer seasons and by 94.91% during the cold seasons in a WSP system, operating in Marrakesh, Morocco. This system consisted of an anaerobic and an aerobic pond in series. They also found that when temperature was increased from 8°C to 23°C, the removal rate of *E. coli* also increased. For *C. perfringens* removal in this study, they were generally tolerant to the wide range of temperature during a 15-day test period.

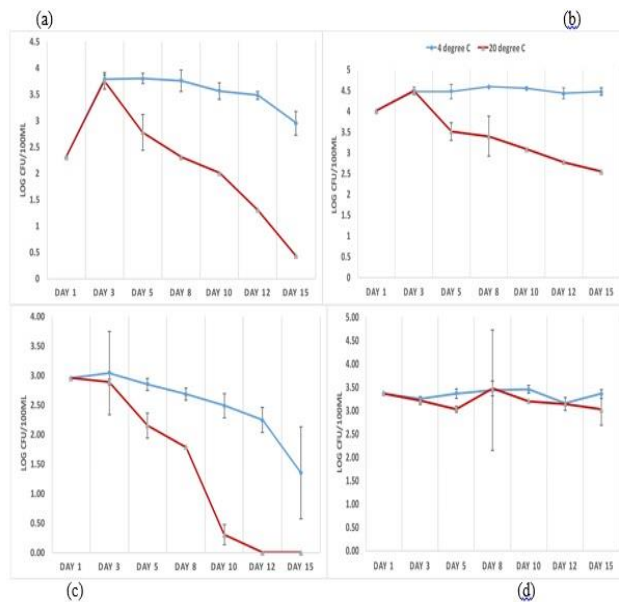


Figure 8. Inactivation and removal of *E. coli* (a), total coliforms (b), *Enterococci* (c) and *C. perfringens* (d) in the secondary effluent wastewater collected from the Alkhademiya sewage station in Baghdad governorate over a 15-day period when they were exposed warm (T=20°C) and cold (T=4°C) temperatures.

4. Conclusion

This paper investigated the effects of pH (5.1, 8.2, 8.4, 9.7 and 10.6), DO (1, 8.5 and 20 mg/L) and temperature (4 °C and 20 °C) on the inactivation and removal of four indicator organisms (two traditional ones and two potential ones). Both algae (*Mougeotia sp.* and *Nostochopsis sp.*) that were collected from the Alkhademiya sewage station in Baghdad WSP were noted to increase both pH and DO when cultivated under laboratory conditions. Their presence is believed to contribute to the high pH and DO levels in the WSP system.

The inactivation of *E. coli* and total coliforms were found to increase with increasing pH. The highest inactivation rates were observed at pH 10.6. Intermediate DO level appeared to support the survival of both *E. coli* and total coliforms, while both high and low DO levels could facilitate their removal and inactivation. *Enterococci* were significantly reduced at both intermediate and high DO concentrations. This would suggest that the presence of algae leading to high DO concentrations could potentially promote the removal of *E. coli*, total coliforms and *Enterococci*.

Two additional indicator organisms (*Enterococci* and *C. perfringens*) showed different inactivation trends compared to traditional indicator organisms. *C. perfringens* were tolerant to the pH, DO and temperature ranges investigated. This would suggest that *E. coli* and total coliforms are limited in their ability to represent the presence and concentration of *Enterococci* and *C. perfringens*. This finding supports the literature where they have been suggested to be potential indicator organisms of pathogens in addition to *E. coli*, fecal coliforms and total coliforms.

Disinfection efficiencies should be expected to exhibit seasonal variations in WSP systems operated in temperate climates. Higher removal efficiencies of indicator organisms would be expected during the warmer seasons, which will continue to pose challenges to winter operation unless some engineered operational modifications can be adapted that will facilitate their retention and removal under colder conditions, which would normally be expected to offer more limited inactivation efficiencies.

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