Microbiological Parameters of Different Waste Waters to Evaluate Their Potential to be Use as a Media for *Chlorella sp. and Spirogyra sp.* Cultivation

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Abstract

Globally there is severe water scarcity, and methods to reuse wastewater need to be adapted. The objective of this research was to determine the microbiological parameters of different wastewaters in order to study the potential of wastewater as a media to cultivate algae.

Food, agriculture, drainage and municipal wastewater sample were collected in sterile bottles separately. Aerobic, anaerobic plate counts, yeast and mold counts were evaluated. Enumeration of coliform, fecal coliform, coliphages and microalgae was performed with all wastewater samples. *Chlorella* sp. and *Spirogyra* sp. were isolated from three different paddy field water samples, and growth studies were carried out in different wastewater media with standard sterile Chu's medium and in sterile tap water medium as control.

Heterotrophic, anaerobic bacteria, microalgae, yeast and molds were found in all types of wastewater. Agriculture wastewater had the highest value for aerobic, anaerobic, yeast and mold count in the highest dilution. Coliforms and fecal coliforms were absent in food and agriculture wastewaters on Eosin Methylene Blue (EMB) medium. Coliform and Fecal coliforms were only observed in drainage and municipal wastewater on Endo agar (EA) medium. Coliphages were absent only in agricultural wastewater and found in the highest numbers in municipal wastewater. Except for agricultural wastewater, other three types of wastewaters contain microbial contaminations. Agriculture wastewater medium significantly enhanced the growth of *Chlorella* sp. than the standard Chu's medium. The growth of *Spirogyra* sp. was stimulated by the food wastewater medium than the other media.

Keywords: Wastewaters, Microbiological parameter, Chlorella sp., Spirogyra sp., Coliforms

1. Introduction

Wastewater contains various pollutants in various concentrations. Wastewaters are created due to liquid and solid waste discharged from residential, commercial properties, factories, agricultural facilities or land. Wastewater is characterized according to its physical, chemical and biological parameters. Physical parameters include turbidity, color, odor, total solids, and temperature. Wastewater contains different chemicals in various forms. Chemical parameters of wastewater are Chemical Oxygen Demand (COD), Total Organic Carbon (TOC), nitrogen, phosphorus, chlorides, sulfates, alkalinity, pH, heavy metals, trace elements and priority pollutants. The biological parameters of wastewater are Biochemical Oxygen Demand (BOD), oxygen required for nitrification and microbial population (Nayla Hassan Omer, 2019, Slavov, Aleksandar Kolev. 2017).

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Untreated wastewater (including feces) contains high numbers of pathogens (Chahal et al., 2016, Cabral, 2010). Therefore, microbiological evidence can be used to indicate that there is a hazard in the environment, but for wastewater, there is no perfect indicator organism because the discharged organisms range from bacteria to worms, protozoa and viruses (Omarova et al., 2016). The most commonly used indicator organisms for monitoring water quality are coliforms and fecal coliforms (Motlagh et al., 2019). The coliforms comprised Enterobacter, Escherichia and Klebsiella, including fecal coliforms, Escherichia coli (E-coli) is the main species (Cabral, 2010).

The treated wastewater is used by people for drinking and other purposes. Therefore, in order to protect public health, wastewater must be treated and then returned to the environment for further use. Wastewater treatment is the process of removing pollutants from wastewater, including physical, chemical, and biological processes to remove these pollutants and produce environmentally safe wastewater. There are several wastewater treatment systems, including conventional treatment systems, macroalgae and microalgae treatment systems etc. (Abdel-Raouf, 2012, Joel de la Noue and Niels de Pauw, 1988). In wastewater treatment systems, bacteria and algae act mutually to decompose organic compounds, absorb nutrients and reduce pollutants (Rishiram et al.,2016, Juan-Pablo Hernandez et al., 2009). Algae produce oxygen, which bacteria can use to break down organic compounds into CO2, which can then use this oxygen to produce more oxygen. Bacteria can also provide important nutrients for microalgae, such as vitamin B12 (Juan Luis Fuentes et al., 2016).

The main nutrient requirements for algae growth are nitrogen, phosphorus and a variety of micronutrients, including potassium (Ayesha et al., 2021). Algae absorb these nutrients and carbon dioxide and produce biomass through photosynthesis.

Municipalities and industries around the world are looking for bioremediation as an important way to deal with wastewater. Bioremediation uses naturally occurring microorganisms (microalgae) to treat nutrients in wastewater (Diana Pacheco et al., 2020). This method provides an effective method that is economically and environmentally sustainable. Algae is a vital bioremediation agent and has been utilized by many wastewater treatments. In an algae-based wastewater treatment system, wastewater treatment and algae cultivation must be considered at the same time. Following parameters are considered and analyzed during wastewater treatment: BOD reduction, Total Dissolved Solids (TDS) reduction, pH, nitrogen removal rate and phosphorus removal rate (Oilgae, 2010, Rejane H. R. Costa et al., 2019, Bunce et al., 2018)

For efficient wastewater treatment, optimum growth of algae needs to be maintained. Compared with the traditional wastewater treatment process, the use of algae is a more economical and effective method to remove biochemical oxygen demand, pathogens, phosphorus and nitrogen (Bulent Sen et al., 2013). The treatment process requires mechanical aeration as aerobic bacteria consume oxygen from the organic compounds in the wastewater.

The algal biomass produces a source of useful products such as biodiesel, bioethanol and biobutanol, and according to some estimates, they can produce large amounts of vegetable oil. Algae can be used to produce hydrogen. Nutrient removal is an important role of algae in wastewater and treated water reused for irrigation purposes, washing, flushing toilets and gardening.

Therefore, the objective of this research was to study the microbiological parameters of different types of wastewater, such as and study the potential of wastewaters used as a media to cultivate *Chlorella* sp. and *Spirogyra* sp.

2. Materials and Methods

Collection of different types of wastewaters
The following four different types of wastewater, such as agricultural wastewater, food wastewater, municipal wastewater and drainage wastewater, were collected in sterile Duran bottles (each 50 mL of three replicates) and immediately transferred to the laboratory to process the microbiological analysis.

2.1. Determination of microbiological parameters
The following microbiological parameters of different types of wastewater samples were determined.

2.1.1. Aerobic plate count
Plate count agar (PCA) plates were prepared under sterile conditions. Water sample was serially diluted up to 10-6 by using sterile 9 mL of saline as a diluent. A sample (0.1 mL) from a particular dilution was taken with a sterile pipette, and it was transferred to the center of the PCA plate. Using a sterile glass spreader sample was spread uniformly over the surface of the medium. Duplicate plates were also made. This procedure was repeated for each dilution, and plates were incubated at 37°C for 24 hours. After incubation, plates were observed for the development of colonies, and the result was expressed in terms of colony-forming units (CFU) per mL of sample.

2.1.2. Yeast and mold count
Yeast Mannitol Agar (YMA) plates were prepared under sterile conditions. Serial dilution of a sample was carried out up to 10^{-6}. A sample from a particular dilution, was taken (0.1 mL) with a sterile pipette, and it was transferred to the center of the YMA plate. By using a sterile glass, a spreader sample was spread uniformly over the surface of the medium and duplicates were also made. This procedure was repeated for each dilution, and plates were incubated at room temperature for 24-72 hours. After incubation (37oC), plates were observed for the development of colonies, and the result was expressed in terms of colony-forming units (CFU) per mL of sample.

2.1.3. Enumeration of coliform bacteria
Eosin methylene blue agar (EMB) plates and Endo agar (EA) plates were prepared under sterile conditions. Serial dilution of a sample was carried out up to 10^{-6}. Samples from a particular dilution were taken (0.1 mL) with a sterile pipette, and it was transferred on the center of the EA and EMB plates separately. By using a sterile glass spreader, samples were spread uniformly over the surface of the media, and duplicates were also made. This procedure was repeated for each dilution, and two sets of plates were prepared. One set was incubated at 37°C, and the other set was incubated at 44°C for 24-48 hours. After the incubation, colonies were counted, and results were expressed in terms of colony-forming unit (CFU).

2.1.4. Enumeration of coliphages
The sample was serially diluted up to 10^{-3}. 1mL of sample form a particular dilution was withdrawn by using a sterile pipette, and it was transferred into the sterile 8 mL of molten agar tube. Young Escherichia coli (E. coli) suspension (1 mL) was added into the same molten agar tube and it was mixed

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well. It was poured immediately on a sterile Petri dish and spreader uniformly by rotating the petri dish. This procedure was repeated for each dilution, and plates were incubated at 37°C for 24-48 hours. After the incubation, the number of clear zones (plaques) was counted.

2.1.5. Enumeration of Microalgae

The standard counting chamber method (Hemocytometer) was used to determine the population of microalgae. This was determined separately for unicellular, colonial and filamentous forms.

2.1.6. Isolation of Microalgae

Chlorella sp. and *Spirogyra* sp. were isolated from the paddy field water sample using the micromanipulation technique. Cells (*Chlorella* sp.) or filament (*Spirogyra* sp.) were transferred into the sterile Chu's medium separately, and they were cultured under continuous aeration with light illumination (Digital lux meter – 3 ½ digit LCD -0.6klx). Finally, pure cultures were obtained by subculturing technique (Chu's medium).

2.2. Growth study of microalgae on different types of wastewater

Salt (chlorine as sodium chloride) in dried fish was determined using AOAC Volhard volumetric method (AOAC, 2005).

2.2.1. Study the growth of Chlorella sp.

Pure Chlorella culture (4.0*104cell/mL) samples (5 mL) were inoculated into 500 mL of sterile Chu's medium, 500 mL of sterile water and 500 mL of four different types of sterile wastewater media separately. These culture media were aerated with light illumination (Digital lux meter – 3 ½ digit LCD -0.6klx). At different time intervals (days), samples were taken from a uniform culture medium, and the cell density was determined by the counting chamber (Hemocytometer) method. This procedure was repeated on each wastewater-based medium.

2.2.2. Study the growth of Spirogyra sp.

Fresh Spirogyra filaments (0.2 g) were inoculated separately into 500 mL of sterile Chu's medium, 500 mL of sterile water and 500mL of four different types of sterile wastewater. These culture media were continuously aerated with light illumination (Digital lux meter – 3 ½ digit LCD -0.6klx).
After 10 days of culturing, fresh weights of the algae were determined by filtering the media through sterile membrane filter apparatus papers separately. The weight difference was calculated.

3. Results and Discussion

3.1. Microbial parameters

3.1.1. Aerobic plate count

Aerobic plate count was counted for all types of wastewaters. The results showed that agriculture wastewater contained the highest number of colonies than the other types of wastewaters. Agricultural wastewater contains 13×106 CFU/mL at the highest dilution factor of 10^{-6}.
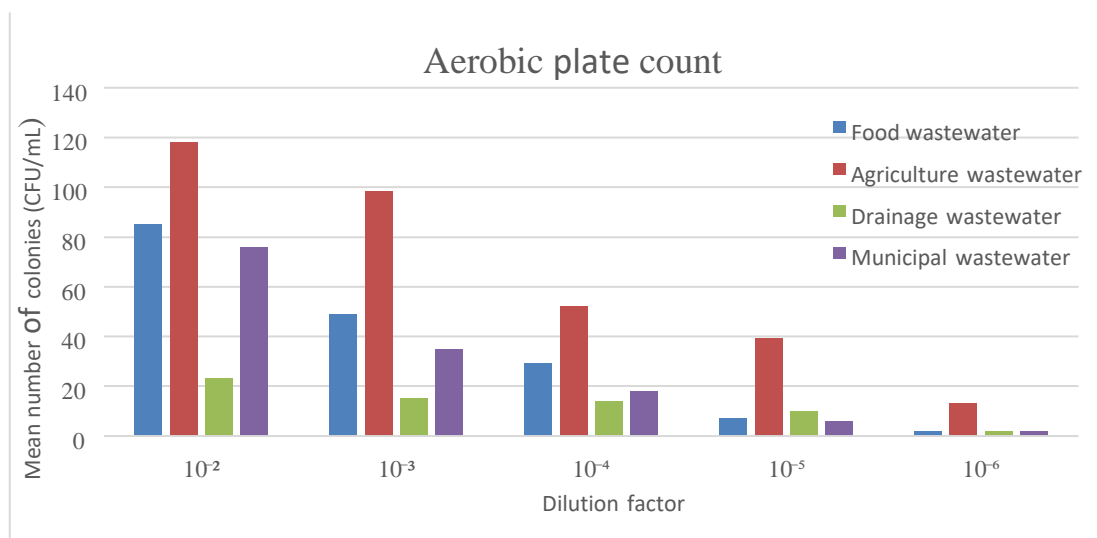
Figure 1. Aerobic plate count in various types of wastewater.

In this study, bacterial counts were high in agricultural wastewater. Aerobic plate count was reported to be ranging from 2.0 to 85×106 CFU/mL in agricultural wastewater. The bacteria can survive and reproduce more successfully under high nutrient levels of nitrogen and phosphorus due to the use of fertilizers.

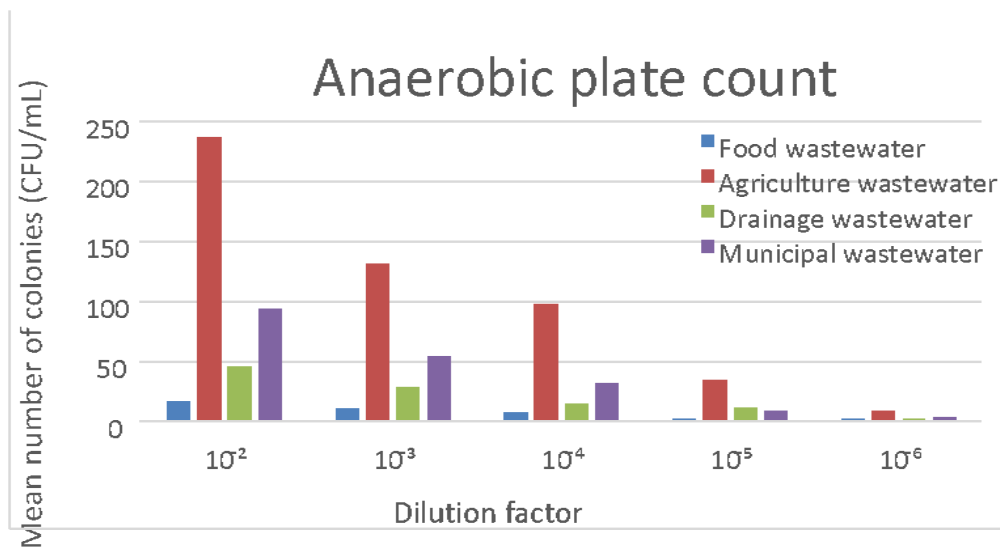*3.1.2. Anaerobic plate count*

Figure 2. Anaerobic plate count in various type of wastewater.

The results indicated that the number of colonies in different types of wastewater was gradually reduced with increasing dilution. Also, the results showed that food wastewater contained the least number

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of colonies compared with other types of wastewater. In the least dilution factor 10^{-2}, food wastewater contained 17×10^{-2} CFU/mL and white colonies were observed.

Statistical analysis indicated that there was a significant difference (α=0.05, P=0.038) among anaerobic plate count of four different wastewaters. The further analysis stated mean number of CFU in agriculture wastewater was significantly different from other wastewater. The reason for the highest anaerobic count in Agriculture wastewater could be due to the use of fertilizers.

3.1.2. Yeast and mold count

The results indicated that the number of colonies in different types of wastewater was gradually reduced with the increasing dilution. Also, the results showed that agriculture wastewater contained the highest number of colonies compared with other types of wastewater. In the highest dilution factor 10^{-6}, agricultural wastewater contained 5×10^{-6} CFU/mL.
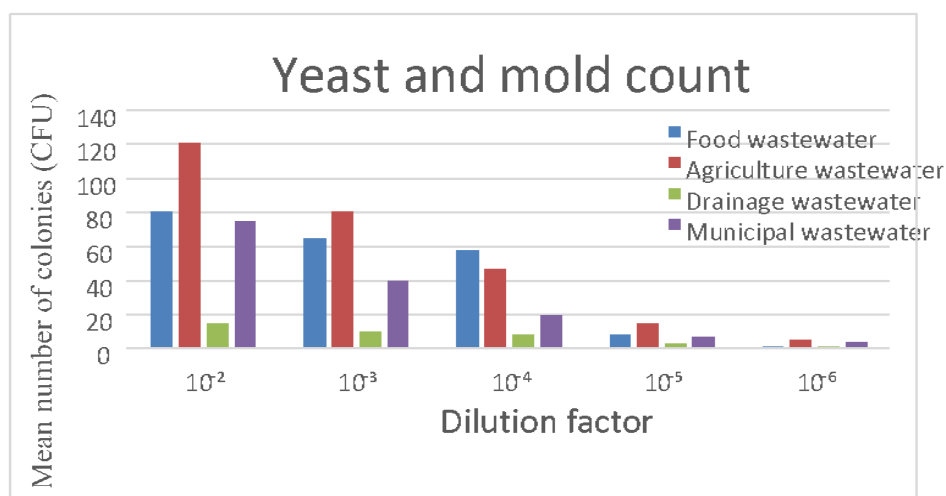

Figure 3 Yeast and mold count in various types of wastewater.

3.1.3. Coliform and fecal coliform count

The results indicated that Coliforms and fecal coliforms were observed in both drainage and municipal wastewaters on EMB medium at 37°C and 44.5°C, respectively. Coliforms were absent in food wastewater, and fecal coliforms were absent in agriculture wastewater on EMB medium with appropriate incubation temperatures. Significant difference was not observed between the count of coliforms and fecal coliforms on EMB medium in drainage wastewater (α=0.05; p=0.191). But the significant difference was exhibited in municipal wastewater (α=0.05, P=0.038). Coliforms and fecal coliforms colonies appeared bluish-black in colour on EMB Medium.

Coliforms and fecal coliforms were observed in both drainage and municipal wastewaters on EA medium, while they were absent in food and agriculture wastewaters. A significant difference was not observed between the count of coliforms and fecal coliforms on EA medium in municipal wastewater (α=0.05; p=0.172). But a significant difference was exhibited in drainage wastewater (α=0.05, P=0.029). Coliforms and fecal coliforms colonies appeared pink or red colour on EA medium. According to the SLS 1246:2003 fecal coliforms should be absent in wastewaters. The results indicated agricultural wastewater was free of fecal coliforms.

According to the national environmental act (No.47: 01.02.2008), the tolerance limit of fecal coliform for the domestic wastewater discharged into marine coastal areas is 60 MPN/100 mL maximum. But in this study, the number of fecal coliforms was 125 CFU/mL, which was extremely, very high.

Tolerance limits of fecal coliform for the industrial wastewater discharged into inland surface waters is 40 MPN/100 mL maximum. But in this study, the number of fecal coliforms was 12CFU/mL, which was extremely high.

3.1.4. Coliphages in wastewater

Coliphages are viruses that infected the E.coli and form plaques, and are used as a fecal indicators. The results showed that coliphages were absent only in agricultural wastewater. Municipal wastewater had the highest number of coliphages. It was also indicated that the coliforms were absent in agriculture wastewater and found in a higher number in municipal wastewater.
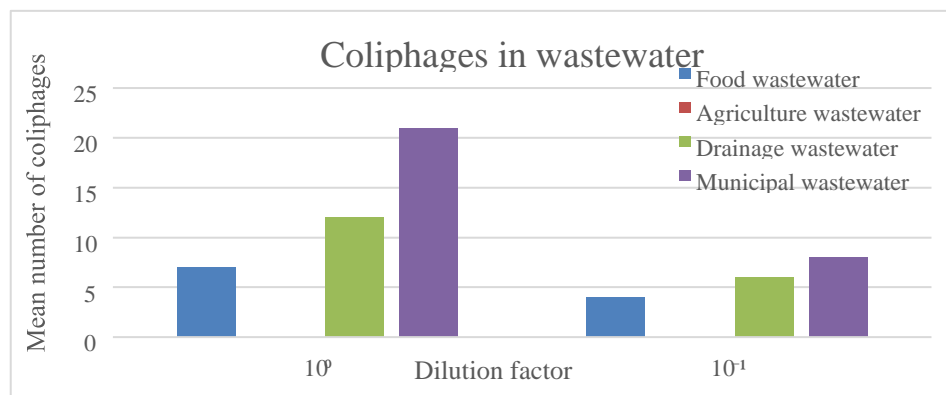

Figure 4. The number of coliphages in various types of wastewater.

3.2 Cultivation of Chlorella sp. in different types of wastewater

All tested four different types of wastewaters were suitable for the growth of *Chlorella* sp. The growth pattern of the Chlorella sp. is similar to the food wastewater medium and the standard Chu's medium. Growth of Chlorella sp. was still in the exponential phase even up to 25 days. But in the standard Chu's medium, the growth of Chlorella sp. reached a stationary phase after 25 days. In the tap water medium (control), growth increased up to 14 days and then gradually decreased. In drainage, wastewater medium growth increased up to 20 days and then decreased, whereas, in municipal wastewater, medium growth increased up to 16 days and then decreased. The typical pattern of growth was observed in drainage wastewater medium and municipal wastewater medium. There was a reduction in cell numbers after particular days in both media due to the removal of nutrients during the growth of Chlorella sp. when compared with standard Chu's medium, the growth of Chlorella sp. was still in the exponential phase up to 25 days. Therefore, the agriculture wastewater medium was the most favorable one compared to other tested media and also the standard medium.

Statistical analysis was carried out, and it exhibited there was a significant difference ($\alpha=0.05$; $p=0.032$) among different types of media. Agriculture wastewater medium was significantly different from other wastewater media. Instead of Chu's medium, agriculture wastewater was recommended to the cultivation of Chlorella sp.
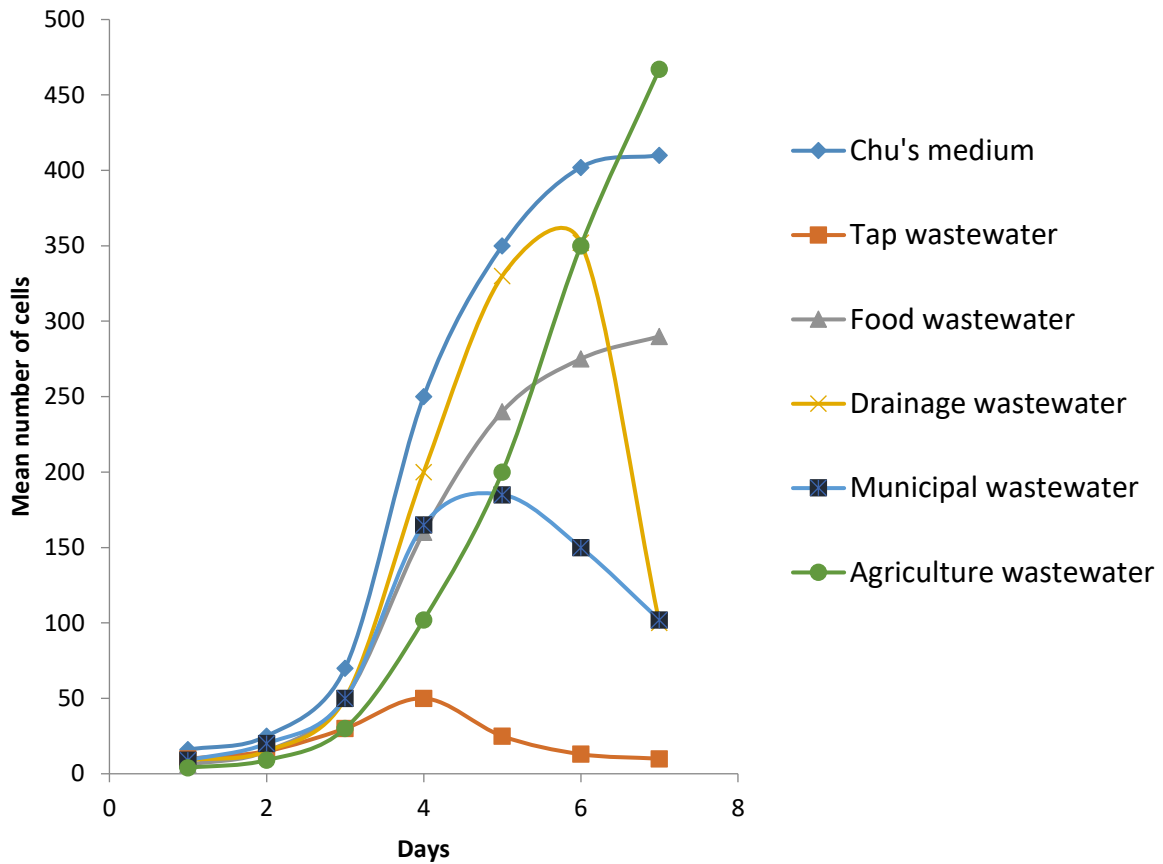
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Figure 5. Growth of *Chlorella* sp. cells in different types of wastewater.

3.2.1. Cultivation of Spirogyra sp.

Growing abilities in food wastewater were significantly better than in drainage wastewater, municipal wastewater and agricultural wastewater. Growth rate of *Spirogyra* in Chu's medium was moderate compared with other wastewater media and less than in tap water. In food wastewater, the growth of *Spirogyra* was the highest amount among all media. Drainage and municipal wastewater showed almost a similar amount of growth as weight differences of 0.625g and 0.514g, respectively. The growth of *Spirogyra* in agricultural wastewater was the least among all media. (weight difference of 0.159g).
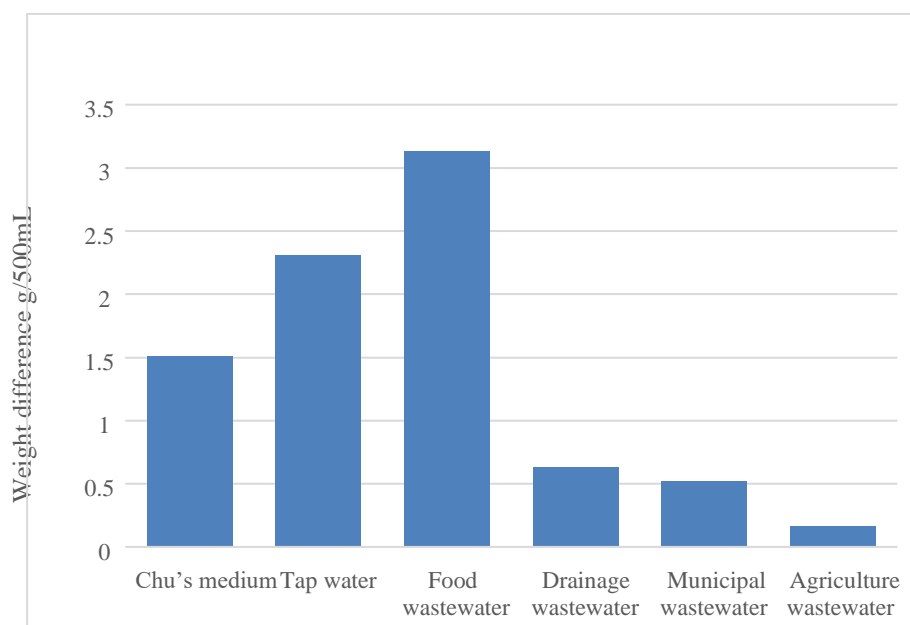
Figure 6. The weight difference of Spirogyra filaments in various media.

4. Conclusion

Heterotrophic bacteria, anaerobic bacteria, a unicellular form of microalgae, yeast and molds were found in all types of tested wastewaters. Total coliforms were only absent in food wastewater, whereas fecal coliforms were only absent in agricultural wastewater. Coli phages were also absent only in agriculture wastewater. Based on the SLS 516: Part 3 (Revised Sri Lanka Standards for Food and Water Microbiology) fecal coliforms should be absent in waste and wastewater. Except for agricultural waste water other three types of wastewater were microbiologically hazardous.

According to the national environmental act (no.47: 01.02.2008), the number of fecal coliforms in the municipal wastewater was extremely very high (125 CFU/mL) compared to the tolerance limit (60 MPN/100 mL maximum), and the number of fecal coliforms in the food wastewater was extremely higher (12 CFU/mL) than the tolerance limit (40 MPN/100 mL maximum)

All tested four media were found to promote the growth of *Chlorella* sp. Agriculture wastewater medium significantly enhanced the growth of *Chlorella* sp. than the standard Chu's medium. The growth of *Spirogyra* sp. was influenced by the food wastewater medium than the other media.

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