

Elimination of *Vibrio Cholerae* from Raw Sewage Using *Chlorella Vulgaris*: A Case of Chegutu Waste Reticulation, Zimbabwe

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ABSTRACT

The research was aimed at eliminating *Vibrio cholerae* from raw sewage collected from home spills of Chegutu, Zimbabwe. An experimental approach was used in which homogenized raw sewage, inoculated with *Chlorella vulgaris* culture was distributed equally into three tanks and left to stand for three weeks. Samples were extracted from the three tanks and tested for the presence of *Vibrio cholerae* and physico-chemical parameters once in seven days. Results showed a continuous decrease in the quantities of *Vibrio cholerae* as well as physico-chemical parameters. *Vibrio cholerae* was totally eliminated from the sewage within 21 days. *Chlorella vulgaris* can be used as a bioremediation agent to eliminate the *Vibrio cholerae* from sewage. The research recommends that the prototype designed from this research could be used at a large scale in the elimination of *Vibrio cholerae* in Municipal raw sewage.

Keywords: *Vibrio cholerae*, *Chlorella vulgaris*, bioremediation, wastewater, physico-chemical parameters

INTRODUCTION

Wastewater and sewage treatment is one of the obligatory services municipalities worldwide have to provide their residents with (Nathanson and Ambulkar, 2018). The wastewater and sewage have to be cleared of any contaminants, impurities and toxic substances before they can be discharged into water bodies that include rivers, dams and oceans, (Maliki et al., 2020). Many developing countries fail to efficiently treat wastewater because conventional plants are too technical and expensive in their construction and maintenance (Wang et al., 2014). These wastewater treatment plants also use expensive chemicals and require massive energy input (Sabuda et al., 2020).

Chegutu sewer pipes were laid more than 50 years ago catering for a smaller population (15,000); however, the population has since ballooned to approximately over 60,000 to date hence the need for a more efficient sewage reticulation system (Chegutu Municipal Newsletter, 2018). The clogged old AC pipes compromise the flow of effluent to the sewer ponds so most residential areas are characterised by frequent bursts, creating a condition of cross-contamination of municipal water, and rendering the water unsafe to drink (Jena and Nyakudya, 2018). **Figure 1** shows the sewage spills at one house in Chegutu town.

Statement of the Problem

In the aftermath of the 2008/9 massive cholera outbreak, the Ministry of Health and Child Care (MoHCC) engaged in various public awareness and health education programmes to conscientise the public on how the cholera bacterium is spread. Some minority groups seem to have not heeded the teachings as witnessed in the 2018 cholera outbreak localized to Chegutu following the passing of a female Moslem due to diabetes mellitus and diarrhoea that was characteristic of cholera (Nyamukondiwa, 2018). As per their tradition, a religious ritual to clean the deceased's intestines was performed. However, the relatives did not wear protective gloves leading to the death of two relatives soon after burial. The deceased exhibited symptoms that include vomiting and watery diarrhoea. The perilous cycle in infections and deaths prompted the MoHCC to intervene and treat it as any emergency (Nyamukondiwa, 2018).

Eleven years after the massive cholera outbreak that claimed 160 lives, sewage spills are still a common feature not only on roads but in residential yards. It is against this background that this research seeks to design a prototype for the elimination of *Vibrio cholerae* from sewage using the microalgae, *Chlorella Vulgaris*. Phytoremediation is a form of bioremediation that makes use of algae, naturally occurring microorganisms to remove pollutants from different types of wastewater (Sharma and Khan, 2013). Microorganisms that have the capability to degrade contaminants by employing



Figure 1. Sewage spills at one house in Chegutu town

metabolic degradation pathways are selected for different bioremediation approaches. In situ, bioremediation methods are preferred to ex-situ because they conserve the natural environment (Kumar and Kundu, 2020). Microorganisms are economical, effective and non-disruptive tools for transforming hazardous waste into less hazardous substances (Kumar et al., 2013).

Objectives

1. Determine the presence of *Vibrio cholerae* in raw sewage spills prominent in Chegutu residential area.
2. Determine methods for elimination of *Vibrio cholerae* in sewage spills.
3. Design a prototype for elimination of *Vibrio cholerae* from sewage spills.

LITERATURE REVIEW

Contained in domestic sewage are arrays of microorganisms that include bacteria, fungi, viruses, and some pathogenic nematodes that originate from soil and sanitary waste (Toze, 1997). Microorganisms present include *Salmonella typhi*, *Vibrio cholerae*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, and *Streptococcus aureus*. They are discharged by persons infected with intestinal diseases such as cholera, typhoid, fever, dysentery and shigellosis (Dumontet et al., 2001).

Sewage treatment is the process of annihilating contaminants, toxins and pollutants from sewage or wastewater so that it can be safely disposed and reintroduced back into the natural environment (Basika et al., 2020). Studies done in South Africa by Okeyo et al. (2018) show that a significant number of wastewater treatment plants release semi-treated effluent that contain pathogens that include

Vibrios. It is imperative that the quality of wastewater effluent is monitored because globally, 80% flows back into the environment (Osunla and Okoh, 2017). The products of sewage treatment are mostly used by humans for various beneficial purposes (Garcha et al., 2016). The liquid effluent may be released into water bodies that include seas, lakes, dams, and rivers or may be used for agrarian purposes while the semisolid sewage sludge may be used as fertilizers. It is therefore important that the wastewater be treated efficiently so that the environment is not harmed by the different microorganisms found in sewage and wastewater (Prasad et al., 2019).

Dasgupta et al. (2019) explain that sewage wastewater treatment is important in reducing the biological oxygen demand (BOD), salinity, chemical oxygen demand (COD), phosphates, nitrates, and heavy metals. These emanate from detergents, softeners, bath soaps, shampoos, and run offs from agriculture. All these reduce the quality of water to levels that are unfit for use and human consumption (Zhao et al., 2016).

Treatment of wastewater is therefore important to reduce pathogens to acceptable levels that do not affect humans, fauna and flora. Treatment methods should aim to destroy most toxigenic pathogens to curb recontamination and remove nutrients such as nitrates and phosphates so that the pathogens will not regrow. Kumar et al. (2013) reiterate that sewage or wastewater effluent may percolate into the ground water resulting in contamination of drinking water. Inefficient treatment therefore may translate to humans and animals being indirectly exposed to toxigenic pathogens that include *S. typhimurium* (Sharma and Khan, 2013).

Demirbas et al. (2017) posit that the nitrates and phosphates in sewage and sewage effluent stimulate growth of aquatic plants and algae which are fed upon by fish. Nongogo and Okoh (2014) further reiterate that elevated nitrates and phosphates further cause increases in fish population which will subsequently impact negatively on the ecological balance. The effects of nitrates in aquatic bodies have been well explained by Salgueiro et al. (2016) who further explain that nitrates may also cause algal bloom which in turn causes oxygen depletion in water bodies; thus, resulting in deaths of aquatic organisms. The abundant nitrates and the phosphates may also cause increased growth of aquatic bacterial populations (Wang et al., 2010). The final effluent may even alter the pH of the receptacle water bodies. These fluctuations in water pH may affect the breeding patterns of the different aquatic organisms. Increases in water pH may also cause bleaching of coral reefs (Olguín, 2003).

Studies have discovered that a number of *Vibrio* species are abundant in effluent from wastewater treatment plants from Sub-Saharan Africa (UNICEF, 2018). Some *Vibrio* species of great medical importance include *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, and *Vibrio cholerae*. All these are toxigenic and are transmitted through contaminated water and food causing serious diseases (Osunla and Okoh, 2017).

Vibrio cholerae are curved, gram negative bacilli and facultative anaerobes that are capable of carrying out both respiration and fermentation (Nongogo and Okoh, 2014). The bacterium is 1-3 micrometres by 0.5-0.8 micrometres and belongs to the *Vibrionaceae* family. It is very motile with a single sheathed polar flagellum (Robinson, 2014). A study

carried out by Huq et al. (2005) conclude that *Vibrio cholerae* are autochthonous to aquatic environments where they thrive on aquatic plant reservoirs that include *Eichhornia crassipes* and aquatic organisms such as crustaceans.

Studies by Bharati and Ganguly (2011) have revealed that toxigenic variants of *Vibrio cholerae*, serogroup O1 and 1039 are the etiological agents of cholera, a gastrointestinal disorder that is characterized by watery diarrhoea, abdominal pains and vomiting. All these symptoms collectively result in severe dehydration that might lead to death. Winstead et al. (2020) explain that the bacteria release an adenosine diphosphate-ribosylating enzyme called the cholera toxin (CT). Bharati and Ganguly (2011) further explain that the CT is the principal virulence factor which increases the pumping out of sodium and chloride ions into the lumen of the small intestines resulting in osmotic imbalance which causes elimination of large volumes of water from the epithelial cells of the small intestines.

Cholera is a grave public health problem in Africa with persistent endemicity as shown by statistics from (World Health Organisation, 2018). Suspected cholera cases reported to WHO by African countries between 1970 and 2014 were 3 221 050. This figure constitutes 46% of all global cases and 56% of these infections were from sub-Saharan Africa (Mengel et al., 2014). Although the most notable cause of cholera and other water borne diseases is contamination of food and drinking water with faecal matter, a number of other factors have also come into play (Cartwright et al., 2013). These include poverty and overcrowding due to natural disasters that force people to stay in refugee camps, political or economic crises, poor sanitation, insufficient health education and ailing economies (Mulamattathil et al., 2014).

Naturally, microorganisms degrade environmental contaminants that are introduced into the environment through natural and anthropogenic processes (Mora-Ravelo et al., 2017). Metabolism of the organic compounds by the microorganisms results in the production of carbon dioxide, methane, water and biomass. The carbon dioxide is used for cell growth and reproduction (Kumar et al., 2013). Salgueiro (2016) explain that the microbes can employ enzymes to transform the contaminants into compounds that are less toxic or harmful to the environment. Bioremediation therefore can be explained as the catabolic potential of a microorganism to degrade pollutants by using them as a source of energy or food resulting in restoration of ecological balance (Mora-Ravelo et al., 2017). Although bioremediation relies on native microbial species, techniques can be employed to enhance the biodegradation potential of the microorganisms. Different microbial species with known degrading abilities could be imported to augment the native species to enhance the degradation process or nutrients and other physical and chemical parameters that promote microbial growth could be added and or adjusted to quantities that will speed up the degradation process (Salgueiro et al., 2016).

Advantages of bioremediation have been explicitly explained by Mora-Ravelo et al. (2017) as being very easy to implement and environmentally friendly because pollutants can be degraded in-situ without disturbing the environment. After the contaminants are changed into innocuous metabolites, the site can continue to be used. In-situ

treatment of contaminants also reduces the risk associated with transportation accidents. The process is more economical compared to conventional methods such as incineration (Mora-Ravelo et al., 2017). However, other researchers that include Sharma and Khan (2013) contend that bioremediation is a slow process that may require close monitoring as contaminants may fail to be reduced to globally acceptable levels. Wang et al. (2010) further contend that although enhancement increases microbial degrading abilities, the substances introduced to enhance the bioremediation process may further cause contamination problems.

Previous studies by Olguín (2003), Sharma and Khan (2013), and Wang et al. (2010) have concluded that bioremediation is dependent upon two factors:

1. the degradability of the contaminant and
2. the site of contamination.

Microorganisms used to detoxify substances that could pose as a danger to man and his surroundings include bacteria, fungi and algae. The use of microalgae to remove pollutants from different environments is known as phycoremediation (Pilon-Smits, 2005).

Chlorella vulgaris is a eukaryotic, unicellular, and photosynthetic microorganisms naturally abundant in aquatic systems that include seawater and freshwater. The microalga has been extensively researched on due to its various and unique properties (Ferreira and Gouveia, 2020). Gonçalves et al. (2017) describe the microalga as versatile with proven efficiencies in nutrition, cosmetics, pharmaceuticals, health, and wastewater bioremediation. Ferreira and Gouveia (2020) further explain that *Chlorella vulgaris* is capable of converting solar energy and carbon dioxide into biomass which in turn can be transformed into diverse marketable products that include food supplements, health supplements, aqua feed, poultry feed, bioacetone, biomethane and biofuel.

Chlorella vulgaris has been employed as a bioremediation agent in different types of wastewater because of its dual purpose of removing pollutants and generating biomass (Sharma and Khan, 2013). Some researchers that have extensively researched on *C. vulgaris* that include Zhao et al. (2016) have concluded that *C. vulgaris* is the best microalga to use for the removal of ammonium in wastewater because it does not generate ammonia, a secondary pollutant. In a study carried out by Taziki et al. (2015), it was revealed that the microalgae were able to remove nitrates and nitrites from wastewater by assimilation. In Zimbabwe, Sero et al. (2021) isolated and selected four microalgae native to Zimbabwe with potential use in biodiesel production. These were *Chlorella spp*, *Oscillatoria spp*, *Scenedemus spp* and *Microcystis spp*. The study revealed that *C. vulgaris* had high growth and lipid production rate.

Microalgae, in general and *Chlorella vulgaris* in general have been used in conjunction with other microorganisms such as bacteria and fungi (Ru et al., 2020). In an experimental investigation to evaluate the performance of *Chlorella vulgaris* and *Enterobacter sp* MN17 to remove organic carbon, heavy metals and colour in wastewater from the textile industry, Mubashar et al. (2020) noted that removal efficiency of *C. vulgaris* increased with addition of an *Enterobacter sp* inoculum. The bacterial inoculation stimulated proliferation

of the microalgal biomass due to the growth promoting characteristics of the *Enterobacter sp.* Bacteria-algae interactions in aquatic ecosystems studied by Cole (2003) showed that bacteria may stimulate algal growth by regeneration of nutrients and production of essential vitamins while algae are capable of promoting bacteria growth by decomposition of organic matter and subsequent release of nutrients.

MATERIALS AND METHODS

The research adopted a quantitative paradigm that was mainly characterized by experimental work. Quantitative research employs the scientific method to systematically investigate and examine the cause-effect relationship by manipulation and controlling of variables. It involves the collection and statistical analysis of numerical data to test hypothesis (Bloomfield and Fisher, 2019; Goertzen, 2017).

Quantitative data was collected by measuring physico-chemical parameters. Untreated raw sewage was collected in a 5-litre sterilized polypropylene bottle to reduce the chances of contamination and possible deterioration. A 2-litre plastic bottle was improvised as a collecting cup by cutting it in half using a surgical blade. The collecting cup was sterilized by washing with hypochlorite and was used to scoop the raw sewage from the ground. Samples were preserved in ice boxes at temperatures around 10°C-15°C within 24 hours. Such ambient temperatures enhance culturability of the microorganism (Sirisha et al., 2017). WHO (2019) states that *V. cholerae* is a biosafety level 2 (BSL-2) pathogen therefore appropriate guidelines for the use and handling of dangerous microorganism were followed.

Data Presentation and Analysis

Data was presented in form of tables showing the growth of *Vibrio cholerae* colonies and various physico-chemical parameters. Physical and chemical parameters that include pH, temperature, salinity, dissolved oxygen, conductivity, turbidity, total suspended solids, total dissolved solids, nitrates, phosphates, chemical oxygen demand and biological oxygen demand were measured immediately upon reaching the laboratory because they affect *Vibrio cholerae* densities (Sharma and Khan, 2013). Prescribed standard laboratory procedures were used to measure different parameters. This ensured validity and reliability of the data.

Physico-chemical parameters were measured in triplicate on the first day and after every 7 days for 3 consecutive weeks. Weekly, 1ml of sewage was also withdrawn from tanks 1, 2, and 3 and subsequently plated onto plates labelled 1, 2, and 3. Colony forming units were also counted from each plate after 24 hours of incubation at 37°C.

Chlorella Vulgaris Culture

The green microalgae strain, *Chlorella vulgaris*, was obtained from University of Zimbabwe, Department of Biological Sciences as a pure culture. The pure culture was grown in Bolds Basal Medium and exposed to light for 5 days to allow the algae to grow. The medium comprised of the following:



Figure 2. Plate 1-*Vibrio cholerae* colonies developing

NaNO ₃	10g
CaCl ₂ .2H ₂ O	1g
MgSO ₄ .7H ₂ O	3g
K ₂ HPO ₄	3g
NaCl	1g

Three erlenmeyer 5-litre containers were filled with 1,000ml of sewage water each and labelled tank 1, 2, and 3. 100ml of *C. vulgaris* culture were later withdrawn and inoculated into each of the three tanks. The 3 tanks were exposed to sunlight during daytime while an external light source, 2×3W fluorescent lamp was availed during the night. Tank tops were covered with aluminium foil to reduce contamination.

RESULTS AND DISCUSSION

TCBS agar plates observed for the presence and identification of *Vibrio cholerae* in sewage showed convincing round, smooth, and slightly flattened yellow colonies that were rather too numerous to be counted. This showed that *Vibrio cholerae* was present in the sewage from Chegutu town. **Figure 2** depicts *Vibrio cholerae* colonies developing.

Tests done by other researchers that include Shaffer et al. (1989) explain that colonies of *Vibrio cholerae* grown on TCBS agar are observed as large (2-4mm), smooth, round, and glistening yellow colonies. At the beginning of the experiment, the agar is blue in colour. Okeyo et al. (2018) explain that this transition from blue to yellow is as a result of sucrose fermentation which increases acidity. It is the acidity which makes the Bromthymol blue, a pH indicator in the medium to turn yellow. The increased pH supports *Vibrio cholerae* growth (Baron et al., 2017). These results from other studies therefore confirm the colonies in plate 1 in **Figure 2** are *Vibrio cholerae* colonies.

Table 1. Parametric measurements at the beginning

Parameter	Reading
pH	7.894
Conductivity (Us/cm)	1,250
TDS (mg/L)	728
Salinity (ppt)	0.5
DO (mg/L)	2.23
Turbidity (NTU)	3,515
TSS (mg/L)	2,150
COD (mg/L)	426
BOD (mg/L)	36
R/Phosphorus (mg/L)	3.604
Nitrates (mg/L)	0.062

These observations were based on the initial physico-chemical parameters as shown in **Table 1**.

The initial measurements of the chemical and physical parameters indicate that the sewage sample contained abundant organic and inorganic nutrients that could be attributed to the elevated *Vibrio cholerae* populations.

Gelatin Agar Results

Suspect isolates from the TCBS agar plates were grown on non-selective Gelatin agar and incubated overnight at 37°C. Smooth, opaque, and white zones around the colonies were observed. This result is comparable with results from a study done in Eastern Mediterranean by Huq and Colwell (1996) who described opaque zone around the colonies as the halo effect.

Gram Staining Results

The Gram staining procedure differentiates bacteria according to their cell wall. Presumptive *Vibrio cholerae* isolates from the TCBS agar plates were stained as morphological confirmation. *Vibrio cholerae* cells appeared comma shaped and stained pink confirming that they are Gram negative which agrees with results by Sirisha et al. (2017). According to Osunla and Okoh (2017) the bacterial cells stain pink because their cell walls contain only 10% peptidoglycan and a greater percentage of lipids. The outer lipopolysaccharide layer is dissolved by the alcohol and they fail to retain the crystal violet colour.

Oxidase Test Results

Gram negative isolates were further selected for the oxidase test which was performed as described by Shields and Cathcart (2010). Colonies were picked with a sterile wooden applicator and streaked on a filter paper saturated with the oxidase reagent. A dark purple colour appeared in 7 seconds confirming that they were *Vibrio cholerae* isolates. This result agrees with observations from Shields and Cathcart (2010).

Presentation and Discussion on Bioremediation of *Vibrio Cholerae*

Results for the bioremediation were presented in two phases, firstly, the physico-chemical parameters and later, the *Chlorella vulgaris* culture effect.

Results of the physico-chemical parameters

Table 2 presents the results of the physico-chemical parameters recorded 7 days after inoculation of the sewage with *Chlorella vulgaris*. Measuring was done in triplicate, from

Table 2. Parametric measurements after 7 days

Parameter	Tank1	Tank2	Tank3	Mean	Change (%)
Ph	8.937	8.762	8.924	8.874	
Conductivity (us/cm)	882	863	849	865	30.8
TDS (mg/L)	521	562	549	544	25
Salinity (ppt)	0.3	0.3	0.3	0.3	40
DO (mg/L)	3.39	2.58	2.29	2.75	23.3
Turbidity (NTU)	1,731	1,843	1,922	1,832	47.9
TSS (mg/L)	951	900	1053	968	55
COD (mg/L)	390	380	431	398	6.9
BOD (mg/L)	32	29	28	29.7	16.7
R/Phos (mg/L)	3.161	3.413	3.408	3.279	9
Nitrates (mg/L)	0.042	0.030	0.033	0.034	45
	0.040	0.029	0.031		



Figure 3. Plate 2-*V. cholerae* colonies 7 days after culturing the sewage with *C. vulgaris*

the 3 Erlenmeyer flasks termed tanks in the table of measurements. Measurement in triplicate was done to ensure validity and reliability.

The first 7 days were characterized by significant changes in the physical and chemical parameter measurements. There was a notable increase in pH and DO. pH increased from 7.894 as shown in **Table 1** to an average of 8.874 (**Table 2**) while DO increased by 23.3% from 2.23 to 2.75 mg/L. However, all the other parameters recorded significant decreases. Conductivity recorded a 30.8% decrease from 1250 us/cm to an average of 865 us/cm while TDS decreased by 25% from 728 mg/L to an average of 544 mg/L. Least decreases were recorded in COD, reactive orthophosphorus and BOD which decreased by 6.9%, 9%, and 16.7%, respectively. TSS showed the greatest decrease of 55% followed with turbidity, nitrates and salinity that decreased by 47.9%, 45%, and 40%, respectively.

Plate 2 in **Figure 3** shows the number of CFUs that had grown on TCBS agar after plating with sewage wastewater 7 days after inoculating it with *Chlorella vulgaris*. Although the CFUs were still numerous, they could be counted with no difficulty using a Gallenkamp colony counter.

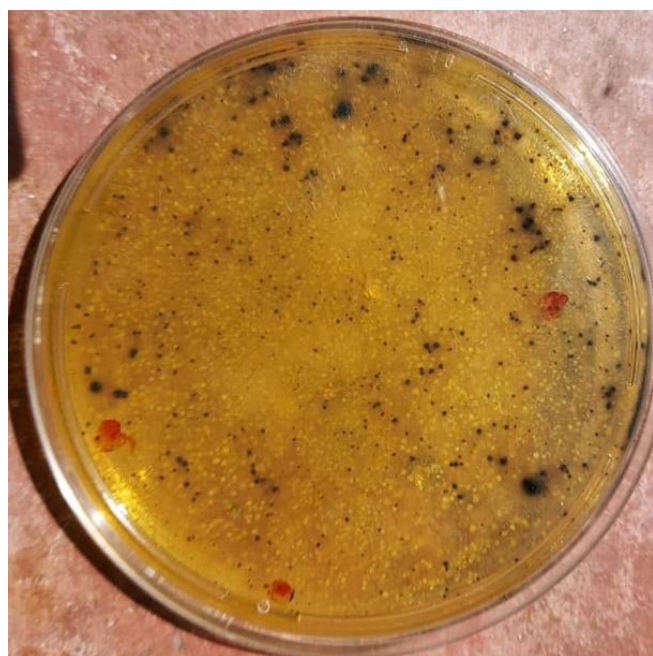
Measurements taken on the 14th day of the experiment (**Table 3**) showed increases in pH and DO. pH further increased

Table 3. Parametric measurements after 14 days

Parameter	Tank1	Tank2	Tank3	Mean	Change (%)
Ph	9.910	9.813	9.752	9.825	
Conductivity (us/cm)	600	637	629	622	28
TDS (mg/L)	364	384	384	377	30.7
Salinity (ppt)	0.1	0.1	0.1	0.1	40
DO (mg/L)	5.3	5.7	5.2	5.4	49.1
Turbidity (NTU)	1,530	1,639	1,701	1,623	11.4
TSS (mg/L)	940	888	1,011	946.3	2.3
COD (mg/L)	351	331	371	363	11
BOD (mg/L)	24	21	21	22	26.7
R/Phos (mg/L)	1.318	1.421	1.444	1.439	56
	1.516	1.440	1.493		
Nitrates (mg/L)	0.031	0.023	0.023	0.025	26.5
	0.029	0.019	0.022		

Table 4. Parametric measurements after 21 days

Parameter	Tank1	Tank2	Tank3	Mean	Change (%)
Ph	8.485	8.606	8.491	8.527	
Conductivity (us/cm)	520	566	417	501	19
TDS (mg/L)	331	345	291	322	14.6
Salinity (ppt)	0	0	0	0	20
DO (mg/L)	3.3	3.7	3.9	3.6	33
Turbidity (NTU)	1,329	1,403	1,433	1,388	14.8
TSS (mg/L)	733	721	825	760	19.3
COD (mg/L)	351	276	172	269	18
BOD (mg/L)	24	21	21	22	17.4
R/Phos (mg/L)	1.318	1.421	1.444	1.439	17.3
	1.516	1.440	1.493		
Nitrates (mg/L)	0.031	0.023	0.023	0.025	39.4
	0.029	0.019	0.022		

**Figure 4.** Plate 3-*V. Cholerae* colonies 14 days after culturing the sewage with *C. vulgaris*

to 9.825 from 8.874 while DO increased by 49.1% to 5.4 mg/L. All the other parameters recorded steady decreases with the least in TSS, turbidity, and COD which decreased by 2.3%, 11.4%, and 11.6%, respectively. Nitrates, BOD, and conductivity decreased by more than 25% while salinity and phosphates decreased the most by 40% and 56%, respectively.

There was not much growth observed in the TCBS agar plates 14 days into the experiment (**Figure 4**). Only three colonies (in red ink) were observed in one plate while the other two plates exhibited no sign of growth. The decline in bacterial population could be attributed to the depletion of essential and key nutrients in the sewage.

All the parameters including pH and DO that had previously been increasing showed moderate decrease in the third week (**Table 4**). DO decreased by 33% to 3.6 from 5.4 mg/L while pH decreased to an average of 8.525 from 9.825. Salts were totally depleted as zero ppt was recorded. Conductivity, TDS, turbidity, TSS, BOD, COD, and reactive

**Figure 5.** Plate 4-*V.cholerae* colonies 21 days after culturing the sewage with *C. vulgaris*

orthophosphorus decreases were below 20%. Nitrates had the greatest decrease of 39.4% to 0.020 mg/L from 0.025 mg/L.

The TCBS agar plates exhibited no sign of growth as evidenced by the colour of the plates (**Figure 5**). There was no sucrose fermentation and therefore no change in pH, hence, the Bromothymol blue, a pH indicator maintained the blue colour. This result shows that the *Vibrio cholerae* in sewage had been totally eliminated.

Table 5 shows weekly average measurements of parameters. Similarly, **Table 6** depicts average CFUs. An average of 506 CFUs were counted in each plate as shown in **Table 6**.

Results from this study have shown that *Vibrio cholerae* was eliminated completely within 21 days of treatment due to the influence of *Chlorella vulgaris*. Maximum reduction in the *V. cholerae* was achieved within the first 7 days of the treatment. Zhao et al. (2016) affirm that *C. vulgaris* is an important player in the management of water resources. Its fast growth rate coupled with the very high proliferation rate has made it the

Table 5. Weekly average measurements of parameters

Parameter	Initial reading	Week1	Week2	Week3	Change (%)
Ph	7.894	8.874	9.825	8.522	
Conductivity (us/cm)	1,250	865	622	501	59.9%
TDS (mg/L)	728	544	377	322	55.8%
Salinity (ppt)	0.5	0.3	0.1	0	100%
DO (mg/L)	2.23	2.75	5.4	3.6	100%
Turbidity (NTU)	3,515	1,832	1,623	1,383	60.7%
TSS (mg/L)	2,150	968	946	763	64.5%
COD (mg/L)	426	398	352	213	50%
BOD (mg/L)	36	29.6	22	18	50%
R/Phos (mg/L)	3.604	3.279	1.439	1.189	67%
Nitrates (mg/L)	0.062	0.034	0.025	0.020	67.7%

Table 6. Average CFUs

Time/days	Colony forming units			Average CFUs/plate
	Tank 1	Tank 2	Tank 3	
0 days	TNTC	TNTC	TNTC	TNTC
7 days	570	470	480	506
14 days	3	0	0	1
21 days	0	0	0	0

microalgae of choice by many in wastewater treatment and pollutant removal.

Results from this study showed that the pH of the sewage water became more alkaline increasing from 7.894 to 9.825 then slightly decreased to 8.525. This result correlates with results from other phycoremediation studies done elsewhere. Studies by Wang et al. (2010) on phycoremediation of wastewater using of *C. vulgaris* concluded that the microalgae experience maximum growth in the pH of range 6.0-9.0. Zhao et al. (2010) who carried out a similar study in China explained this increase in pH as a result of a shift in chemical equilibrium by the *C. vulgaris*. This occurs when carbon dioxide combines with water resulting in the formation of carbonic acid. The carbonic acid later disintegrates into a carbonate and a hydroxyl group. It is therefore the increase in carbonate concentration coupled with the decrease in carbonic acid that increases the pH of the sewage water. Chiu et al. (2015) explain the increase in pH as being caused by the degradation of organic compounds by *C. vulgaris*.

Reactive orthophosphorus decreased by 67% from the initial 3.604 mg/L to 1.180 mg/L during the study period. In other studies, done by Wang et al. (2010), *C. vulgaris* was able to remove 97% of the phosphorus from wastewater. In another study by Salgueiro et al. (2016), *C. vulgaris* removed 99.2% phosphorus from synthetic wastewater. The microalga assimilates the phosphorus into its cells and use it to synthesize different components of the cells that include phospholipids, Adenosine triphosphate (ATP) and nucleic acids. The element is also believed to be important in cell division (Zhao et al., 2016). Studies by Mohammed et al. (2014) have also revealed that microalgae in general and *C. vulgaris* in particular can employ the "luxury uptake" mechanism to assimilate and store phosphorus. *Chlorella vulgaris* have high phosphorus removal efficiency because they have high sequestration potential therefore they can sequester the element from the phosphates in the sewage and use them for growth and biomass accumulation (Dasgupta et al., 2019).

Study by Xin et al. (2010) concluded that the concentration of phosphorus in wastewater is directly proportional to *C. vulgaris*' growth.

Nitrates were reduced by 67.7% from the initial 0.062 mg/L to 0.020 mg/L. Shahid et al. (2020) posit that just as in phosphates, *Chlorella vulgaris* assimilate nitrogen into their cells and use them as nutrients resulting in growth and increase in biomass. Gonçalves et al. (2017) explain that nitrates are actively taken into the algal cells via the cell surface membrane. In an experimental study by Kumar et al. (2013) to test the nitrate removal efficiency of *C. vulgaris* from aqueous solutions, the microalgae removed 88% of the nitrates. In another study by Otondo et al. (2018), *Chlorella vulgaris* achieved a 70.3% nitrate removal. The 67.7% nitrate removal achieved in this study is therefore coincident with results from other researches. However, in other studies, *C. vulgaris* achieved more than 90% nitrogen removal. A study by Sharma and Khan (2013) on bioremediation of sewage wastewater using selective algal species, *C. vulgaris* achieved a 90% nitrate removal. This figure is however not in sync with findings from the current study. This could have been caused by the elevated pH which affects nitrate assimilation. Segovia-Bifarini et al. (2020) explain that ammonium is a form of nitrogen preferred by microalgae because very little energy is used in ammonium assimilation as compared to nitrates. However, in pH above 8, the ammonium disintegrates to form ammonia, a form of nitrogen toxic to microalgae. In this study, pH increased to 9.825 so it can be inferred that the *C. vulgaris* could not efficiently assimilate the nitrogen and phosphorus due to ammonia toxicity (Kumar and Kundu, 2020).

A study by Wirth et al. (2020) revealed that both nitrates and phosphates consumption in wastewater largely depends on how easily light can penetrate. Turbidity, TSS TDS therefore affects removal of these inorganic compounds. The slight decrease of 9% in phosphorus during the first 7 days of the experiment could be attributed to the high turbidity. Initial turbidity reading was 3,515 NTU which decreased by almost 48% in the first week to 1,832 NTU. This sharp decrease in turbidity also resulted in a sharp decrease of 56% in phosphorus in the following week. These results strongly correlate with conclusions from the above study by Wirth et al. (2020). TSS and TDS were reduced by 64.5% and 55.8% respectively in the current study. The decrease in the suspended and dissolved solids could be explained as a result of the *C. vulgaris* sequestration efficiency. The total dissolved and suspended solids are nutrient rich therefore they are taken in by the microalgae for growth (Gonçalves et al., 2017). Studies by Sharma and Khan (2013) using different microalgal strains that included *Chlorella minutissima*, *Scenedemus spp*, and *Chlorella vulgaris* showed an 90% average T, A. LDS removal by all the microalgal strains. TSS value is generally increased by the presence of organic and inorganic matter in sewage (Wang et al., 2010). The 64.5% reduction was therefore as a result of utilization of the organic and inorganic compounds (Segovia-Bifarini et al., 2020). In another study by Kumar et al. (2013), *Chlorella vulgaris* reduced TDS by 97.5%. The slightly lower TSS and TDS removal efficiencies exhibited in this study are not comparable with results from previous studies. This could be as a result of the high pH (9.825) that

could have affected the microalgae. Most microalgal systems operate best at pH of 7.0-8.0 (Zhao et al., 2016).

In this current study, the DO of the sewage increased gradually to 100% with the increase in experimental time from 2.23 mg/L to 5.4 mg/L. Cooper and Smith (2015) assert that this increase in DO is as a result of photosynthesis where oxygen is generated as a by-product. Depletion of *Vibrio cholerae* populations in the sewage wastewater denotes that fewer organisms were using up the oxygen hence the increase in DO (Ajala and Alexander, 2020). Cooper and Smith (2015) explain that as the pH increases, the DO also increases and they attribute this increase to microalgal photosynthesis which produces oxygen molecules. Another study by Segovia-Bifarini et al. (2020) revealed that DO is affected by salinity. Increased salinity in sewage wastewater results in decreased DO because saltier water holds less oxygen. In this study, salinity decreased by 100% from 5 ppt to 0 ppt thereby conforming to findings by (Segovia-Bifarini et al., 2020).

COD gradually decreased during the experimental period by 50% from 426 to 213 mg/L due to the *C. vulgaris*' assimilating potential. The increased assimilating potential of organic compounds results in the decrease in COD concentration. *Chlorella vulgaris* is capable of utilizing both organic and inorganic carbon as an energy source and as a substrate (Segovia-Bifarini et al., 2020). Researches done by Kumar et al. (2013), Olguín (2003), and Sharma and Khan (2013) all showed that most microalgal species that include *Chlorella vulgaris*, *Chlorella minutissima*, and *Scenedesmus spp* have high COD removal efficiencies. However, Wang et al. (2010) contend that COD removal efficiency depend on the wastewater type and range from 50.9%-83%. The 50% decrease in COD is therefore in sync with results from other studies. COD removal efficiency can be as low as 38% as was discovered by Mohammed et al. (2014) when they cultivated *C. vulgaris* in textile wastewater.

BOD was reduced by 50% from 36 mg/L to 18 mg/L. The reduction in BOD could be attributed to the ability by *C. vulgaris* to use up organic compounds for their metabolic processes. *Chlorella vulgaris* is able to efficiently utilize carbon, hence the increase in BOD consumption, (Berthold et al., 2019). Mohammed et al. (2014) used locally isolated *C. vulgaris* to treat wastewater and in that study, BOD and COD were reduced by 70% and 55%, respectively. Findings from this research are therefore consistent with findings from researches done elsewhere. However, in other studies, higher BOD quantities have been removed using the same *Chlorella vulgaris*. Rothermel (2011) coupled wastewater treatment with an algal photo bioreactor and BOD was reduced by 83%. Using results from their research, it can therefore be inferred that *C. vulgaris* could not further metabolise the organic compounds in the sewage without any outside influence as was the case with the photobioreactor (Rothermel, 2011).

Vibrio cholerae populations were totally eliminated within the 21 days of the study period as shown in **Table 5**. A study by Cole (2003) to investigate interaction of bacteria and algae revealed that algae are capable of inhibiting bacterial growth in two ways. Algae can produce a number of polyunsaturated fatty acids that have antibiotic effect. This was reiterated by Fergola et al. (2007) who explained that chlorellin is one of the compounds produced by algae to stifle growth of bacteria.

Chlorellin affects both Gram positive and Gram negative bacteria by disrupting important life processes that include respiration and cell division. Cole (2003) further posits that as the algae grow, the rate of photosynthesis also increases resulting in depletion of organic compounds and elevated pH. The elevated pH alters the macro-environments and decreases the nutrient removal abilities of the bacteria. In another study by Mubashar et al. (2020) to investigate the effect of algae on coliforms and *E. coli* in seafood wastewater, it was observed that bacterial population were significantly reduced in 3 days. Ru et al. (2020) further explain that competitive interactions inhibit bacterial growth because algae outcompetes the bacteria in assimilation of limiting nutrients such as nitrogen and phosphorus. In the current study, *Vibrio cholerae* thrived in parametric conditions expressed in **Table 1**, as the nutrients decreased in quantities, the *V. cholerae* also reduced in numbers. It can be concluded that the change in physical and chemical parameters caused by *C. vulgaris* competitively excluded the *Vibrio cholerae*. Results from a study by Wirth et al. (2020) on microalgae-bacteria interactions in wastewater concluded that bacterial species diminish because of the high dissolved organic and inorganic removal capabilities of the microalgae. This result, therefore, strongly correlates with results from other researches globally.

Description of the Prototype

The design of the prototype follows the findings from the research and the conditions under which the findings were obtained. Therefore, the following prototype design was constituted based on (i) physico-chemical parameters and (ii) microalgae-bacteria relationship. This prototype was done at reduced settings therefore bioremediation at larger scales require the multiplier effect. The prototype is presented in the form of a flow diagram (**Figure 6**).

In the flow diagram, to the raw sewage distributed into the three tanks, an equal amount of *C. vulgaris* culture was added and the contents were homogenised. Extracts from each tank were taken and physico-chemical parameters were measured and recorded on weekly basis. *V. cholerae* were also enumerated. The bioremediation capacity of *C. vulgaris* resulted in the progressive reduction in colony counts of the *V. cholerae* as the experiment progressed, until such a time when no colonies were identified in the samples NB. The different colour intensities on the flow diagram from deep to light blue signify the bioremediation effect of *C. vulgaris*.

CONCLUSIONS AND RECOMMENDATIONS

Samples of raw sewage taken from home spills in Chegutu contained large quantities of *Vibrio cholerae* that were too many to be counted. This may explain why there has been high prevalence of cholera outbreaks in the town. The physical and chemical parameter tests carried out on the sample revealed high quantities of phosphates, nitrates, organic and inorganic compounds. Literature from past studies explains that it is these elevated quantities of pollutants that cause *Vibrio cholerae* to thrive in the sewage wastewater.

Chlorella vulgaris proved to be the microalgae for the bioremediation of the *Vibrio cholerae* in the raw sewage

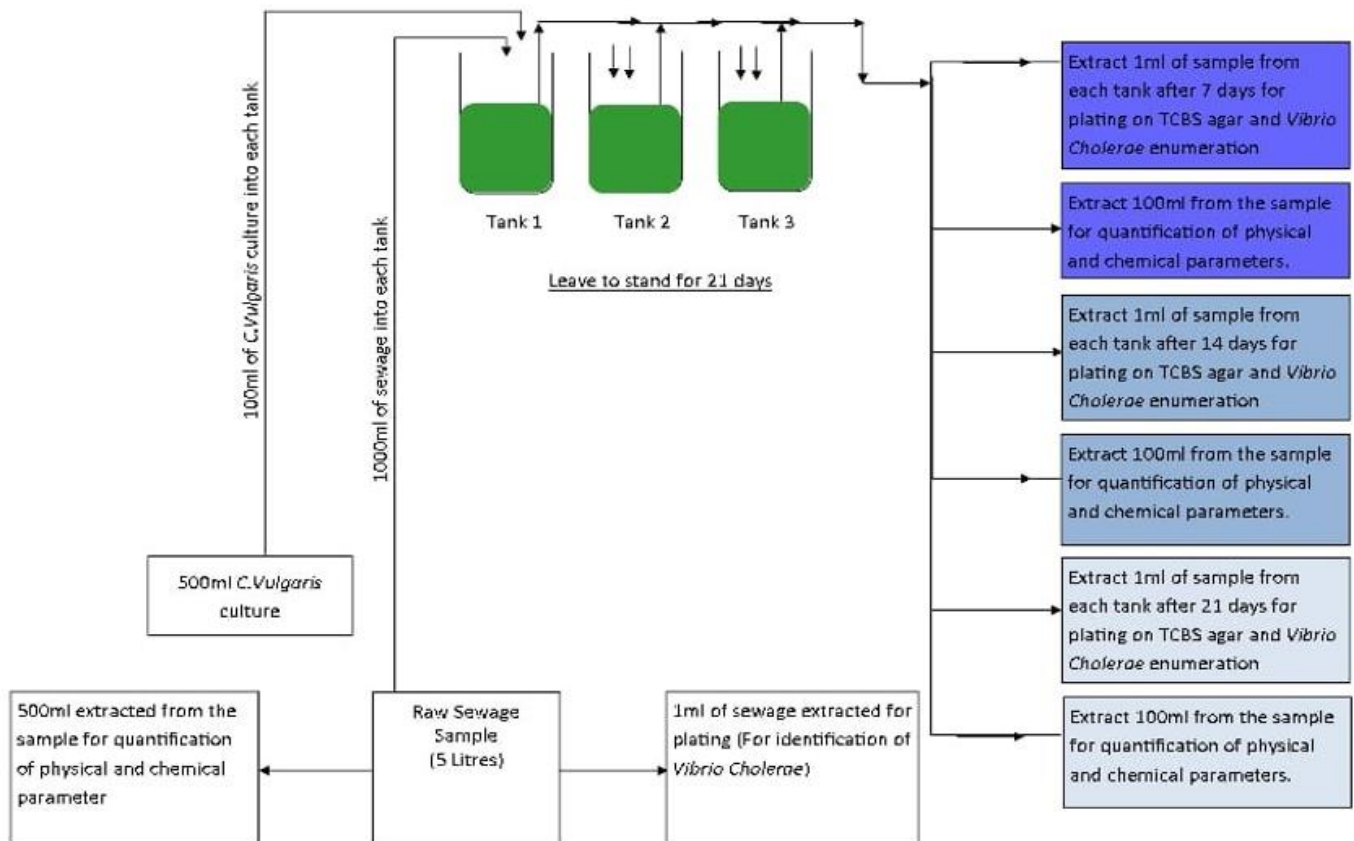


Figure 6. Prototype for the elimination of *V. cholera* from Chegutu town raw sewage

sample. The microalga's capacity to remove nutrients efficiently as explained in previous studies helped to achieve the bioremediation process. The proliferation of the microalga in the nutrient rich raw sewage helped in the elimination of the *Vibrio cholerae* coupled with the reduction in the physico-chemical properties of the raw sewage. Indirectly this has made the sewage less polluted and hence prevents eutrophication. Whilst this method achieved the total elimination of the *Vibrio cholerae*, there was reduction in the level of pollutants in the sewage.

Chlorella vulgaris demonstrated that it is pollutant sequestrant therefore the decrease was due to the microalga taking up the pollutants and use them as nutrients for growth and biomass increase. The nitrates, phosphates, total dissolved and suspended solids are assimilated by the microalgal cells and used for synthesis of cell components that include phospholipids. The gradual decrease in COD and BOD denotes that the pollutants in the sample were depleting. Continued removal of these inorganic compounds by the microalga eventually leaves the wastewater fit for reuse especially in agriculture. The increases observed in DO and pH were both as a result of photosynthesis. *Chlorella vulgaris* had proliferated due to the nutrients in the sample resulting in increased photosynthesis rate and subsequent increase in the quantities of oxygen produced. The DO value increased because there was less *Vibrio cholerae* in the sample to take up the oxygen for respiration.

Depletion of pollutants, organic and inorganic compounds disrupt the natural phycosphere throwing the *Vibrio cholerae* in states in which they fail to assimilate the phosphates and the nitrates. The *Chlorella vulgaris* outcompete the *Vibrio*

cholerae in assimilation of nutrients. This is in line with previous studies that showed that the microalga is capable of producing polyunsaturated fatty acids that exhibit antibiotic properties thereby inhibiting growth of *Vibrio cholerae*.

Following the outcome of the results obtained from laboratory procedures, a prototype has been designed for the bioremediation of sewage from Chegutu Municipality. Whilst the municipality has its traditional way of sewage treatment, the designed prototype could augment this by eliminating *Vibrio cholerae*.

The research findings paved way for the design of a prototype to eliminate *Vibrio cholerae* from Municipality of Chegutu sewage. The prototype could be employed to eliminate other microorganisms that cause different microbial infections in communities. These microorganisms include *Salmonella spp*, *Shigella spp* and *Campylobacter spp*.

The prototype produced in this current study was done at laboratory scale; however, it could be used at a larger scale using the multiplier effect. As alluded to in the introduction section of the first chapter, the town of Chegutu has experienced a couple of cholera outbreaks due to contamination of drinking water with sewage spills that are common in streets and some homesteads. The prototype could therefore be adopted to eliminate *Vibrio cholerae* and pollutants from these sewage spills.

The *Chlorella vulgaris* prototype could be used by municipalities for the treatment of sewage wastewater to halt eutrophication that can be caused by the phosphates and nitrates that may be contained in chemicals used in wastewater treatment plants. The phosphates and nitrates

may cause algal blooms in water bodies resulting in death of fish and other aquatic organisms. *Chlorella vulgaris* could therefore serve dual purpose of eliminating both microorganisms and chemicals accumulated in water bodies.

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