ROLE OF *Chlorella vulgaris* Beijerinck IN THE REMEDIATION OF WASTEWATER AND AS ANTI-BACTERIAL AGENT

Simranjit Kaur Virdi

M.Sc. (Botany), Mount Carmel College, Autonomous

Bengaluru

Abstract

The aquatic ecosystem balance is upset today because of population explosion and its influence in and around freshwater reservoirs that largely declines the quality of water- its physical, chemical and biological properties; thereby causing adverse effect to human health and the overall aquatic ecosystem. The present study was based on concocting a phycoremediation technique, by using the microalgal strain *Chlorella vulgaris* Beijerinck to reform wastewater into reusable form. For this purpose, the microalga was cultivated at room temperature with artificial illumination and aeration, in the surface water collected from Hulimavu Lake, situated in Bengaluru, India, that is laden with nitrogen, phosphorous and chlorine. To assess the quality of surface water of the lake, eight parameters were analyzed: pH, turbidity, dissolved oxygen, chloride, carbonate, phosphate, nitrate, and sulfate content. The study showed a positive relation between an increase in algal growth with increase in dissolved oxygen level, and a decline in the levels of chloride, phosphate, carbonate, and nitrate; for a total bioprocess time of 15 days. The ethanolic extract of the green microalga was also tested under the cultured conditions by adopting the standard agar well diffusion method against two Gram-negative bacterial strains *Escherichia coli* and *Klebsiella pneumoniae* that are commonly found in wastewater, carrying several water borne diseases. As per the growth rate assessment of *Chlorella vulgaris* and while testing its microbial inhibition character, it is concluded that this algae is having the ability of remediating polluted surface waters of its inorganic nutrients and pathogenic bacteria. Subsequently, the efficiency of phycoremediation was further improved by immobilizing *Chlorella vulgaris* in beads of sodium alginate. On evaluating and demonstrating the effect of immobilization of algae in remediation of wastewater, efficiency in the technique and an advantage over the use of conventional suspension culture was obtained.

Keywords: *Chlorella vulgaris*, remediation, phosphate, surface waters, Hulimavu Lake, anti-microbial agent, immobilization.

Introduction

Water is imperative to the existence of all life forms. However, the sustainability of this renewable resource is questionable as the demand for potable water increases today with exponential population growth and the need of high quality water for domestic purposes and economic activities. Among the various environmental challenges that India faces today, water scarcity ranks the highest. India, being an economy in transition from a developing to a developed nation, faces the problem of lack of management and infrastructure for the expanding inhabitants. The urban population in India has jumped from 25.8 million in 1901 to 1.3 billion (estimated) in 2017. This has thrown up two self-perpetuating problems, viz. shortage of water and sewage overload [53]. In this context, adequate and clean water supply, sanitation measures and management of sewage and solid wastes becomes a major concern. To deal with this, majority of the towns and cities do not have efficient sewerage and sewage treatment services. Cities have expanded beyond municipalities, but the new urban agglomerations remain under rural administrations, which do not render the capacity to handle sewage in a sustainable form. The sewage is either directly dumped into rivers and lakes or in open fields.

This indiscriminate disposal of industrial and municipal wastes, leads to heavy pollution of water, threatening all life forms. Therefore, it is vital to focus on techniques to partially, if not fully, remove contaminants (nitrates, phosphates and heavy metals) from wastewater, before discharging them into waterways.

1.1. Conventional Wastewater Treatment

According to U.S. geological survey, it is projected that by the year 2025 global water demand will exceed supply by 56% [53]. To solve this water crisis and to meet the growing demand of freshwater, wastewater treatment techniques focussed at remediating wastewater should be employed. Wastewater can be defined as any type of water who's physical and chemical properties have been adversely affected due to anthropogenic influence. It is foreseen that by 2050, about 132 billion litres of wastewater per day, with a potential to meet 4.5% of the total irrigation water demand would be generated [53].

There are broadly two classes of wastewater i.e. municipal wastewater and industrial wastewater. Sewage is a type of municipal wastewater characterised by physical, chemical and bacteriological toxicity. Industrial wastewater comprises of organic, inorganic and heavy metal constituents rendered useless during a manufacturing process. Thus, an overview of water resources indicates that in the coming years, there will be a twin edged problem to deal with reduced fresh water availability and increased wastewater generation due to increased population and industrialization [54].

Central Pollution Control Board (CPCB) studies depict that there are 269 sewage treatment plants (STPs) in India, of which only 231 are operational. Thus, the treatment capacity is just 21% of the present sewage generation. In class I cities of India (New Delhi, Mumbai, Chennai, Kolkata and Bengaluru) activated sludge process is the most commonly used technology to remediate wastewater, and in class II cities (Jaipur, Agra, Lucknow, Chandigarh and Nagpur) Common Effluent Treatment Plants (CETPs) have been set up as cluster of small scale industries [55] wherein the municipal and industrial waste is initially collected and subjected to primary and secondary treatment processes.

During the primary process, screening and aeration techniques are used to remove large solids (sand, glass pieces etc.) from the wastewater. After this primary process, the wastewater yet contains high amount of organic, inorganic and heavy metal constituents that is dealt with in the secondary treatment process. In this step, the organic content of the wastewater is bio-oxidised by micro-organisms within aeration tanks. This produces a residual semi-solid material called 'sludge', that settles at the bottom of the treatment plant. The sludge is pumped into a 'digester' or 'sludge thickener' to release carbon dioxide and methane gas. However, the residual sludge is composed of undetected pathogens, untreated organic and inorganic micro-pollutants and heavy metals that have escaped the degeneration process. Further, the discharged water leaving the secondary treatment plant contains significant levels of phosphorous and nitrogen. Moreover, in such conventional processes there is no special method employed during the treatment cycle to decompose heavy metals from wastewater.

Therefore, there is an urgent need of a paradigm shift from the current complex and inefficient wastewater treatment protocol to a sustainable, eco-friendly and cost-effective management of wastewater.

1.2.Bengaluru's Dying Lakes

Most of the sewage generated in urban cities is discharged directly into storm water drains that directly link to water bodies because of the lack of adequate infrastructure for sanitation and inappropriate waste management practices. And nowhere is it more evident than in Bengaluru, once known for its beautiful lakes. Bengaluru is located on a ridge with natural water courses along three directions viz. the Vrishabhavati, Koramangala–Challaghatta and Hebbal–Nagavara valley. These form important drainage courses for the interconnected lake system which carries storm water- misused for waste disposal, beyond the city limits. Today, this untreated sewage has progressively consumed the lakes of Bengaluru, resulting in a) algal blooms; b) proliferation of aquatic weeds and macrophytes; c) large scale death of fish due to asphyxia and; d) frothing due to phosphorous enrichment [57].

Of the three major catchments, the Koramangala–Challaghatta valley houses the largest of the water, and Hulimavu Lake (Table 1.1) is one of its feeders, contributing to shedding ~45% of the city sewage over the last 60 years into the catchment area. Unplanned urbanisation and increasing developmental activities around the lake area (Fig. 1.1 and 1.2) have resulted in eutrophication, DO depletion, malodour, and an extensive growth of water hyacinth that covers 70-80% of the lake in the dry season. Such problems have been recurring despite the fact that certain part of the sewage undergoes at least primary treatment [55].

Name of the lake	HULIMAVU LAKE
Geographic details	12°52'13"N 77°36'18"E
Location	Hulimavu, Bannerghatta Road, Bengaluru, Karnataka 560076, India.
Area	44.26 Ha
Status	POLLUTED
Restoration	No
Water Condition	POOR (Class 'E')

Table 1.1: Geographical and ecological status of Hulimavu Lake[56].

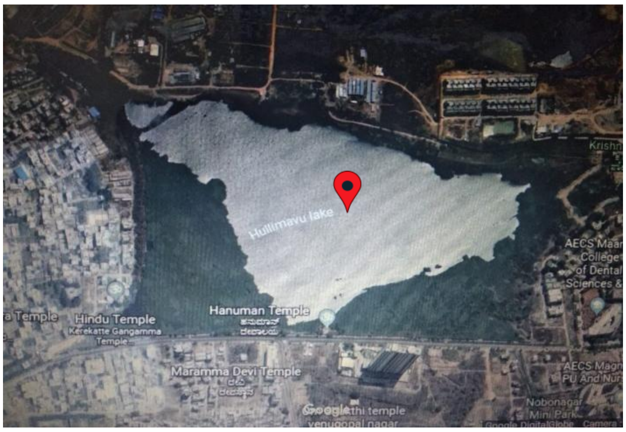
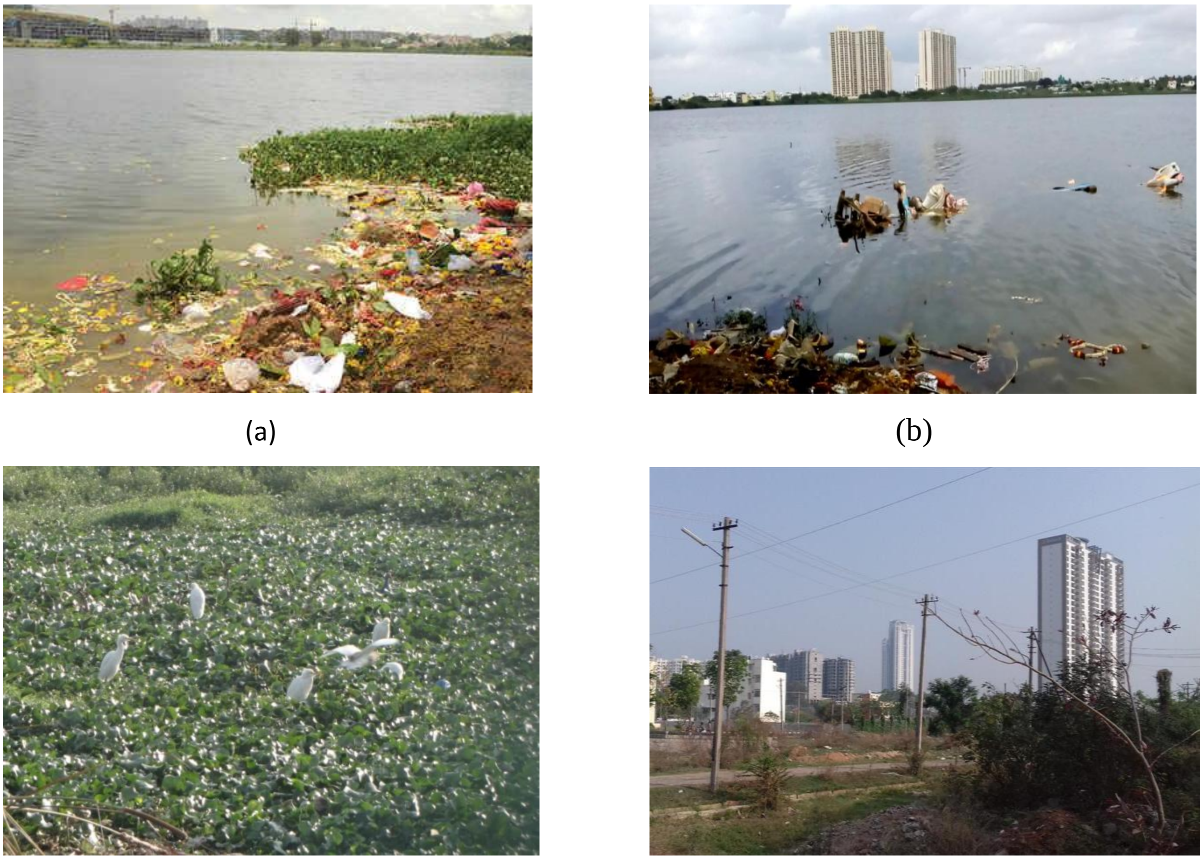


Figure 1.1.: Google Earth image of Hulimavu Lake as of 7th March 2019.



(a) (b)

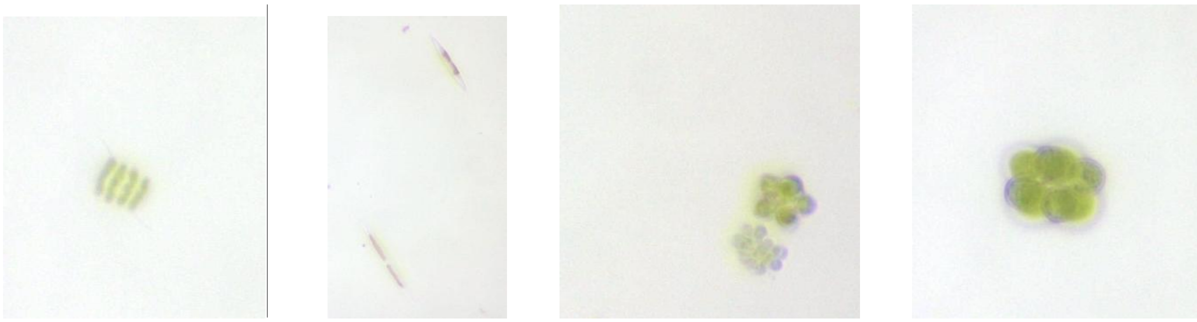
(c) (d)

Figure 1.2: Local human disturbances in and around Hulimavu Lake[56] (a) Unmanaged waste disposal in the lake; (b) Immersion of idol; (c) Macrophyte cover on the lake; (d) Unabated construction around the lake

Sewage brings in large quantities of carbon, nitrogen and phosphorous into the lake ecosystem. Carbon (C), rendered as dissolved carbon dioxide and soluble carbonates and bicarbonates causes hardness of the water. Nitrogen influent in sewage is organic nitrogen (N), urea, ammonia, nitrites and nitrates. Higher concentrations of nitrates and phosphates primarily contributes to the nutrient enrichment of this lake and causes phenomenal alteration to the integrity of the aquatic ecosystem, resulting in deterioration of the ecosystem and at the same time resulting in degradation of water quality, thereby inviting associated health risks.

1.2.1. ***Sources of nitrogen and phosphorous:-*** Discharge of sewage is the major source of nutrients in the lake. Moreover, fertilizers of largely nitrogen (N) and phosphorous (P) which are designed to boost plant productivity end up destroying the water quality. The general urban run-off brings in detergents which increases hardness of the lake water because of the incorporation of sulphates and carbonates.

1.2.2. ***Effects of nitrogen and phosphorous contamination:-***The continuous flushing in of untreated residual water from the surrounding households (Fig. 2d) is accelerating the death of the lake biodiversity via eutrophication. The encroachment of Hulimavu Lake by shooting levels of organic and inorganic nutrients not only causes algal blooms and changes in species composition, but also increases the water turbidity, super-saturation of DO in the daytime, and development of resistant bacteria and algal species (Fig. 1.3)



(a) (b) (c) (d)

Figure 1.3: A few algal species found in Hulimavu Lake water, as observed under 45X (a) *Scenedesmus* sp.; (b) *Closterium* sp.; (c) *Gleocapsa* sp.; (d) *Chlorella* sp.

Toxic compounds produced by these resistant organisms make their way into the food chain, resulting in animal mortality and spread of various water borne diseases like cholera, diarrhoea, gastroenteritis etc to humans. KSPCB claims Hulimavu Lake water to fall below the 'E' category in terms of water quality i.e. declaring it unfit for any use at all- domestic or agricultural.

1.3.Need for Novel Technology

Further, it is to be emphasized that Bengaluru city is not on the banks of any perennial river. It receives water from Cauvery River, and the demand for water is increasing day by day [7]. Therefore it is necessary to treat the waste water generated by households and industries, and recycle it for irrigation, toilet flushing, industrial cooling and other secondary purposes.

Conventional methods of wastewater treatment consume a great deal of energy, use chemicals like chlorine and ozone for disinfecting the water and have high operational costs. Inadequate disinfection processes result in various micro-organisms that eventually develop resistance (AMR) to disinfectants and continue to multiply in partially treated effluents making their way into the environment. Additionally, these methods generate toxic sludge which brings us back to the question of efficient toxic-removal technology.

On account of the depleting natural resources, there is an urgent need to bring up a sustainable, eco-friendly, energy efficient and cost-effective technology for remediating wastewaters. By introducing an alternative biological method (bio-remediation) for the treatment of nutrient rich wastewaters, containing high amounts of nitrate and phosphate, most of these issues can be mitigated. Bioremediation is a process that deliberately uses organisms (micro-organisms or plants) to detoxify contaminated lake or soil conditions to its original condition.

1.4.Role of Microalgae in Remediation of Wastewater

Microalgae are autotrophic, photosynthetic, unicellular, microscopic organisms typically found in freshwater systems, individually or in groups. Unlike higher plants, microalgae or microphytes do not have roots, stem or leaves and are specially adapted to an environment dominated by resistive forces. Algae consume carbon dioxide, nitrogen and phosphorous as major nutrients and require Ca, Mg, Na and K as macronutrients, releasing approximately half of the atmospheric oxygen.

Therefore, wastewater is a good source of the required nutrients for microalgae cultivation. During the growth period of algae, they not only accumulate nutrients for self-perpetuation, but also enhance the growth of bacteria used in degrading organic matter. Hence, the use of algae in remediating wastewater (phyco-remediation) can prove to be an alternative method to the conventional wastewater treatment techniques; for not only is it feasible to grow microalgae in wastewater treatment plants but also, the obtained algal biomass can be further processed to extract the accumulated compounds for secondary purposes such as, biofuels, manure, and bioactive compounds. The present study focuses on the potential of one such green microalgae strain (Chlorophyceae) *Chlorella vulgaris* Beijerinck, in remediating nitrate and phosphate laden wastewater from Hulimavu Lake, at a physical, chemical and biological level.

1.5. Logic of Strain Selection

To accomplish a bioremediation process using microalgae, selection of the algal strain is a key consideration. Algae are a diverse group of aquatic, eukaryotic, organisms that have an estimated 10 million species on Earth; contributing to almost half of the atmospheric oxygen. Extensive research has been directed since the 1980's to identify the algal strains suitable for wastewater treatment [57].

Complimenting this, the current research work is focussed on one such species *Chlorella vulgaris* Beijerinck, which has been found to grow well is wastewater. This particular microalgal strain was selected for the present study as it is very robust compared to other microalgal species and can grow dynamically in presence of essential nutrients which can be readily obtained from any kind of wastewater. It also holds the ability to remove heavy metals from its nutrient media [23]. *Chlorella vulagris* can tolerate a wide range of growing conditions such as, varied nutrient concentrations, temperature and pH making it versatile for wastewater remediation. In addition, *Chlorella* sp. can grow in all three growth conditions (autotrophic, heterotrophic and mixotrophic) and accumulate nutrients up to 60% of its biomass [58] and consequently release bioactive compounds such as amino acids, polysaccharides, lipids and carotenoids for sustenance. These compounds can be further processed for secondary purposes such as for bio-fuel and bio-fertilizer production, in pharmaceuticals and neutraceuticals.

Research Objectives

2.1. Broad Objectives

Traditional wastewater cleansing techniques are prohibitively expensive and tedious to operate and manage. This has necessitated research into developing alternative treatment techniques that are sustainable and at the same time, effective in the overall remediation of the wastewaters with the least possible generation of toxic compounds. The primary objective of this study was focussed on evaluating and demonstrating the potential of microalgae for bioremediation of wastewater laden with nitrogen (N) in the form of nitrates, phosphorous (P) in the form of phosphates, carbon (C) in the form of carbonates, chlorine (Cl) in the form of chlorides, and sulphur (S) in the form of sulphates. The secondary objective was to look into the foremost problem of harvesting the algal biomass from remediated wastewater as well as frame a strategy for maximum remediation, based on the experimental results, which can be integrated into the existing wastewater treatment facilities.

2.2. Specific Objectives

- To evaluate the growth of *Chlorella vulgaris* in wastewater samples taken from Hulimavu Lake.
- To determine the rate of nutrient removal (phosphates, nitrates, chlorides, carbonates and sulphates) by *Chlorella vulgaris* from actual wastewater obtained from Hulimavu Lake.

- Study the inorganic nutrient removal mechanism of *Chlorella vulgaris.*
- Investigate the capability of the selected microalga in rejuvenating the water system by increasing the DO content.
- To evaluate the anti-microbial activity of the selected algal strain against disease causing bacterial strains *Escherichia coli* and *Klebsiella pneumoniae*, commonly found in wastewaters.
- Determine the requirements of improvising an algal treatment system within a conventional wastewater treatment plant.
- Design a technique suitable for easy collection of treated algal strain from wastewater treatment systems.
- Propose and design a commercial based algae wastewater treatment technology (AWT) which mainly focuses on providing a phyco-remediation technique that requires low maintenance and has minimal complexity and operator requirements for treating wastewater containing inorganic nutrients.

Review of Literature

Population explosion and urbanisation has deteriorated the quality of freshwater reservoirs around the world to such an extent that today, some 80% of the world's wastewater is discharged untreated into the environment; causing serious impact on human, animal and aquatic life. Organic and inorganic substances thereby released into the environment as a result of domestic, agricultural and industrial activities have lead to pollution. This can be attributed to the excessive discharge of nitrogen and phosphorous through domestic, agricultural, sewage water and industrial effluents.

The concept of using algae in water treatment was introduced by Oswald and Gotaas (1955) [1] in the paper "Photosynthesis in Sewage Treatment". Microalgae are employed as a tertiary treatment process to remove nitrogen and phosphorous from wastewater [2], since they require nitrogen, phosphorous, carbon dioxide, and light for their autotrophic metabolic growth [3].

Cultivation of algae was started as early as in 1970s, in wastewater ponds to treat secondary effluents so as to prevent eutrophication [4]. In recent years, microalgae bio-treatment has been coupled with the production of potentially valuable biomass, which can be used for several purposes, such as in food supplements, pigments, pharmaceuticals and biodiesel production. On this stance, algae are employed as renewable natural biomass (they form the world's largest group of primary producers, responsible for 32% of global photosynthesis) and as biosorbent material [5] for detoxifying and remediating polluted water. For this purpose, 'phycoremediation' *viz*. the use of micro- and macro-algae for reduction or bio-transformation of pollutants [6, 7] has gained immense popularity.

Most of the bio-treatment technologies use bacteria, but implication of microalgae for effluent treatment, either as single species of *Scenedesmus*, *Chlorella* or *Spirulina*[8-11] or as mixed consortia [10] to correct nitrogen, phosphorous and chemical oxygen demand (COD) from different types of effluents; has already gained momentum.

Discussing the potential removal of phosphate, nitrate and nitrite using freshwater green algae *Chlorella vulgaris* and *Gleocapsa gelatinosa*, Dominic VJ, Soumya Murali and Nisha MC (2009) reported [12] efficient reduction in nutrient load of the water sample within 25 days of study. In a demonstration given by P. Hanumatha Rao *et al*. (2011) [13] in the bio-treatment of effluents from a leather processing facility, situated at Ranipet, Tamil Nadu, using microalga *Chlorella vulgaris* showed that *C. vulgaris* exhibited discernible nutrient scavenging properties, under both laboratory and field conditions. In an effort to phycoremediate IARI's sewage wastewater with *Chlorella minutissima, Scenedesmus spp.* and *Nostoc spp.*, Gulshan Kumar Sharma and Shakeel Ahmad Khan (2013) observed [14] that *C. minutissima* withheld the best remediation potential. It recorded the highest uptake of nitrogen and phosphorous content from the water sample, and hence has the highest manurial potential. According to V.V. Pathak, D.P. Singh, R, Kothari and A.K. Chopra (2014) phycoremediation of textile wastewater using *Chlorella pyrenoidosa*[15] essentially reduced pollutant load and caused decolourization of textile wastewater. Kannan (2011)[16] reviewed the detoxification capacity of a variety of microbes, especially cyanobacteria. It showed that *Anabaena flos-aquae* can serve as a potential bioremedial organism for industrial pollution based on the analysis of its photosynthetic pigments and nitrogen status, before and after treatment.

Among the several microalgae used to treat effluents, *Chlorella sp.* has been found to grow in mixotrophic environment [17]. *Chlorella vulgaris* has also proved to be potent in the removal of ammonia and phosphorous, during the biological treatment of secondary effluents from an agro-industrial based wastewater of a dairy industry [18]. The pH increase which is mediated by the growing algae facilitates phosphorous precipitation and ammonia stripping to the air, disinfecting the wastewater of its phosphorous and nitrogen content [19]. By kinetic investigations, conducted in 2012[20] revealed that the bio-sorption process was greater for *C. vulgaris* than *Dunaliella salina* and so, *Chlorella* sp. would be a potential candidate for the management of industrial wastewater.

The benefits of using microalgae in nutrient biosorption include: i) low operational cost; ii) faster growth rate (as compared to higher plants); iii)large surface to volume ratio; iv) ability to bind to nutrients and metals up to 10% of their biomass; v)possibility of recycling assimilated nitrogen and phosphorous within the algae biomass as manure; vi)avoidance of sludge handling problem; vii)no synthesis required; viii)useful in both batch and continuous systems [21] particularly justifies the worthiness of microalgae as phytoremediators.

Algae liberate oxygen from water as a by-product of photosynthesis. This oxygen is used by bacteria to aerobically bio-oxidise the organic compounds present in the wastewater and subsequently boost their population. An end-product of this bio-oxidation is carbon dioxide which is fixed by the algae during photosynthesis [22]. This cooperation between microalgae and bacteria results in the remediation of organic and inorganic waste from polluted water from toxic to non- or less toxic values. At the same time, microalgae biomass can definitely be maximized and bacterial growth increased.

At this conjecture, untreated water not only causes eutrophication, but is also a major health hazard because of the likely presence of various kinds of pathogens [23]. The danger is serious when domestic and industrial wastes are mixed. In this case, the levels of pollutants get amplified with time, which ultimately leads to shift of bacterial and algal populations towards more resistant species.

Bacteria provide the largest component of microbial community in all biological wastewater treatment processes and range in numbers of 10^6bacteria/ml of wastewater [24]. According to the World Health Organisation (WHO), the mortality of water borne diseases exceeds 5 million people each year, owing to various anthropogenic activities. Government data shows, over the five years- to 2017, India registered 69.14 million cases of water associated diseases such as cholera, diarrhoea, typhoid, and viral hepatitis; with diarrhoea being the second leading cause of death after cholera. Pathogenic organisms of concern in wastewater carrying such diseases include bacteria such as *Salmonella* sp. (typhoid fever and other serious salmonellosis), *Shigella* sp. (bacillary dysentery), *Klebsiella* sp. (pneumonia and urinary tract infection), pathogenic strain of *Escherichia coli* (acute diarrhoea and gastroenteritis), *Yersinia* sp. (plague), *Campylobacter* sp. (dysentery and abdominal pain with fever), *Vibrio* sp. (Cholera) [25, 26] viruses and protozoa. Their cosmopolitan distribution, low dosage requirement to induce infection and long persistence in the environment makes these organisms a significant problem in contamination with wastewater, mainly in rural areas [27]. Moreover, presence of tough walls makes these microbes extremely resistant to disinfection with chlorine and monochloramines [28].

The burden of prevailing infectious diseases has created a significant need for antimicrobial therapy and hence our widespread dependency on antibiotics. However our interactions with the microbial world, further complicates the issue because the common bacteria have steadily developed resistance to these antibiotics, resulting in antimicrobial resistance (AMR). Although the majority of deaths related to antibiotic resistance occur in hospitals, antibiotic-resistant infections can happen anywhere, and are most common in the general community. (US Centre for Disease Control and Prevention, 2013).

The progression of AMR occurs when a drug is no longer able to inhibit or control the action of a micro-organism (bacteria, fungus, virus or parasite) that was previously sensitive to it. As such, the usual treatment regime is no longer effective, and the resistance of pathogens to existing antibiotics has become a global epidemic. The demonstration of pandemic and multi-resistant *Escherichia coli* clone B2-O25b-ST131 being frequently detected in treated wastewater, accentuates the risk of transmission of clinically important multi-resistant microbial strains through wastewater. Therefore, there is continuous research on the field to limit the use of antibiotics.

Modern screening methods have isolated antibacterial compounds in the secondary metabolites of algal members belonging to Phaeophyceae, Rhodophyceae, Chlorophyceae, Chrysophyceae and Bacillariophyceae [29]. Impediments to algal survival such as, exposure to osmotic stress, UV light, high levels of oxygen and salinity (often met through polluted wastewaters), triggers the algae to produce antibacterial compounds[30] identified as fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing heterocyclic compounds, carbohydrates and phenols [16]. High rate algal ponds and stabilization ponds follow basically the same remediation mechanisms and are highly efficient according to fecal bacterial indicators.

Hence, besides removing pollutants from mixed domestic-industrial wastewater, microalgae have the ability to easily acclimatize to extreme environments and produce many valuable compounds. Bactericidal substances were first isolated in 1944[31] when benzene and fatty acid extracts of *C. vulgaris*, were found to inhibit *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyrogenes* and *Bacillus subtilis*. Santoyo S. *et al.* in 2009[32] worked on antimicrobial activity of ethanolic extracts of *Haematococcus pluvialis* against *Escherichia coli* and *Staphylococcus aureus.* Similarly, in a screening conducted by Younes Ghas emi *et al.* in 2007[33], *Chroococcus dispersus, Chlamydomonas reinhardtii* and *Chlorella vulgaris* appeared to be the most promising strains against *E. coli, Salmonella typhi, S. auereus* and *P. aeruginosa.* Antimicrobial activity against *Klebsiella pneumoniae, Bacillus cereus*, and *Micrococcus luteus* was investigated by Neelam Arun, Shalini Gupta and D.P. Singh in 2017[34] using the methanolic extract of *Chlorella pyrenoidosa, Nostoc muscorum,* and *Spirulina platensis.* Antibacterial activity of *Scenedesmus sp.* isolated from a natural pond against water borne pathogenic bacteria has been documented. Vishnu and Sumathi in

2014[35] specially worked on the activity of *C. vulgaris* against pathogenic bacteria found in water such as *Klebsiella sp.* and *Vibrio sp.* One of the antibacterial substances, named “chlorellin”, extracted from the microalga *Chlorella* spp. was found to exhibit inhibitory effect against both Gram-positive and Gram-negative bacteria [31].

In this sense, biological wastewater treatment setups with microalgae have particularly gained importance from 1957 until now, and it is today widely accepted that algal wastewater treatment systems are as effective as conventional treatment systems. Some microalgae species particularly *C. vulgaris* and *Spirulina spp.* are today not only used as potential agents against pathogens associated with water borne diseases, often carried through polluted wastewater; but also employed in skin care products owing to their anti-microbial potency[36].

However, one of the major and practical limitations faced in algal treatment systems is harvesting or separation of algal biomass from the treated water discharge. A competent removal of algal biomass is needless to say, essential for re-cycling of the wastewater. Numerous efforts have been devoted to establish a decisive technology for harvesting microalgae ranging from simple sand filtration techniques to energy-intensive centrifugation [37]. Auto-flocculation, i.e., self-aggregation by ceasing aeration followed by decantation, particularly for cyanobacteria, has also been practised. Harvesting microalgae with ultrasonic waves has been experimented by *Bosma et al* [38]. In this context, immobilization of algal cells for wastewater treatment has been proposed for circumventing the collection problem while retaining the high-value algal biomass for further processing [39]. An immobilized cell is defined as a cell that by natural or artificial technique is restricted from moving independently of its neighbours to all parts of the system under study [40].

Application of immobilization technology to algal wastewater treatment provides more flexibility in the reactor design when compared with conventional suspension methods. This can be attributed to the accelerated reaction rates due to an increased cell density, increased cell wall permeability, and better operational stability over their free-living counterparts [41]. The first paper involving the study of immobilized algae was published in 1969 by Hallier U.W. *et al.*[42] who studied photosynthetic light reactions in chemically fixed *Chlorella pyrenoidosa.* Since then, immobilization strains of microalgae have been used for sewage treatment and production of fine chemicals.

The need for immobilization was underscored by Travieso in 1992[43], when he was able to demonstrate the efficacy of depuration using a fluidized bed of *Chlorella vulgaris.* Garbisu *et al.* (1994) [44] studied phosphate removal efficiency of foam immobilized *Phormidium laminosum*. Soon after, Tam *et al.* in 1994 [45] used *Chlorella vulgaris* cells immobilized in alginate for removing nitrogen and phosphorous from wastewater and they achieved significant reductions in the levels of ammonia and phosphate in wastewater. Similar results were obtained from immobilized algal cells of *Scenedesmus dimorphus* by Gonzalez *et al.*, in 1997 [46]. In subsequent studies by Mallick in 2002 [47], it was observed that immobilized algae with a cell density of 0.1g dry wt./L was the most efficient for nutrient and metal removal in a pH range of 6.0 to 8.0, and chitosan could be a promising algal- support matrix for water detoxification. Later, Murugesan *et al.* in 2003 [48] and Shashirekha *et al.,* in 2008 [49], worked on immobilized cells of *Spirulina platensis* for remediation of wastewaters.

In recent years, Dubey *et al*[50]., worked with immobilized cells of *Oscillatoria* sp., *Nostoc* sp., *Synechococcus* sp., and *Nodularia* sp. in 2011, giving results of removal of nitrate, organic and inorganic phosphate percentage ranging between 91.6-100%, indicating the potential of natural resources as efficient agents for pollution control. Further work was devoted to immobilization of algal cells of *Dunaliella salina* in decreasing the amount of chemical oxygen demand (COD) and phenolic compounds from olive mill waste water. Similar results were demonstrated by Kshirasagar in 2013 [51], and a recent work conducted on similar lines [51] using immobilized *C. vulgaris and C. minutissima* cells to remediate wastewater of its BOD, COD, phosphate and nitrate levels demonstrated significant improvement in the quality of water.

As we can see, microalgal treatment of wastewater has been investigated for over five decades as of now, as an alternative to remove nutrients and heavy metals from wastewater, as well as, control the spread of microbial contaminants such as bacteria, carrying water borne diseases. Several cyanobacterial and chlorophycean soecies have been employed and identified as a working model for such studies because of their expeditious ammonium and nitrate uptake, phosphate removal, reduction in the COD levels, and heavy metal removal; influencing the biological, physical and chemical properties of sewage effluent with high growth rate of algal biomass. Their capability of multi-contaminant removal can be greatly enhanced using efficient algal strains in fixed applications, as biofilms or immobilized cells- usually as consortium to reduce the treatment time and better remediation action.

The present work is an effort to evaluate the potential of chlorophycean microalga *Chlorella vulgaris*, commonly found in freshwaters, to phycoremediate polluted wastewaters, using the strategy of immobilization, against inorganic nutrients discharged due to anthropogenic activities; as well as demonstrates a promising strategy for the replacement of antibacterial chemicals for correcting such wastewater.

Materials

4.1. Wastewater Sample

To study the role of *Chlorella vulgaris* in the treatment of wastewaters, untreated wastewater samples were collected from Hulimavu Lake, Bengaluru (Fig. 4.1.). Standard techniques of sample collection were followed as listed in [59].

Figure 4.1: Site of wastewater collection from Hulimavu Lake

4.2. Microalgae

The microalgae selected for this study, *Chlorella vulgaris*, was obtained from Indian Agricultural Research Institute (IARI), Bengaluru. The stock culture was received in agar slants from the source; and stored in aseptic conditions at room temperature (22°C) and illuminated with bright white fluorescent light. For the purpose of study, the algal strain was enriched in Bold Basal Medium (BBM).

4.3. Growth Medium

The enrichment medium used for *Chlorella vulgaris* was Bold Basal Medium (BBM); the composition of which is listed in Table 4.1.

CONSTITUENT	CHEMICAL FORMULA	QUANTITY (per 100ml)
Sodium nitrate	$NaNO_3$	0.25gms
Sodium chloride	$NaCl$	0.025gms
Calcium chloride	$CaCl_2$	0.025gms
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	0.075gms
Potassium dihydrogen phosphate	KH_2PO_4	0.175gms
diPotassium hydrogen phosphate	K_2HPO_4	0.075gms

Table 4.1: Composition of Bold Basal Medium (BBM).

Six stock solutions of 100ml each were employed; each containing one of the above mentioned salts in the amount specified therein. 10ml of each of these stock solutions was added to 940ml of distilled water. The medium was sterilized by autoclaving at 15lbs pressure and 121°C for 15mins and plugged.

4.4. Culture conditions

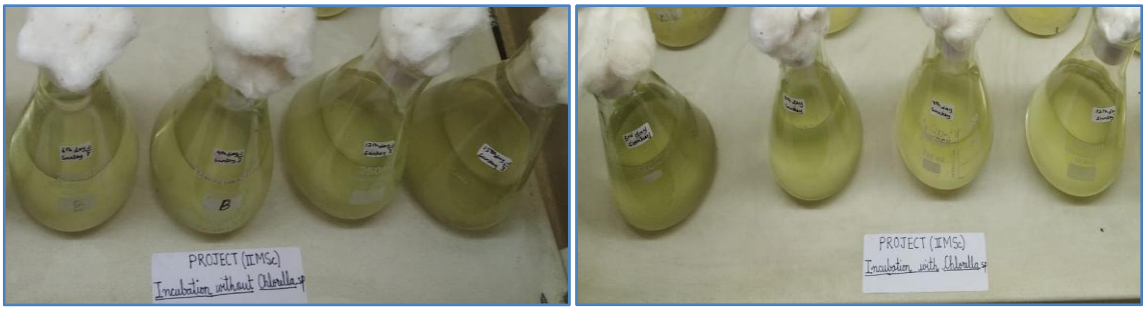
To produce sufficient material (algae) for the wastewater treatment experiments, stock culture (or 'mother' culture) was grown in 500ml Erlenmeyer flasks containing 250ml of BBM at 22±2°C with cool white fluorescent lights giving a continuous irradiance for about 10hours, and plugged securely with cotton to allow infusion of air into the flask. After 2-3 weeks, when the algae had reached exponential growth phase, part of it was harvested by centrifugation at 3000rpm for 10mins and was used in wastewater and anti-bacterial experimental studies.

4.5. Experimental Setup

Before the start of any experiment, all glass apparatus were washed with phosphate-free, non-foaming liquid cleanser, scrubbed with a nylon brush to remove any stains and then rinsed thoroughly with distilled water. Glass apparatus used for anti-microbial study was sterilised by autoclaving at 15lbs pressure and 121°C for 15mins.

The same experimental setup was used for assessing the inorganic nutrient removal from the wastewater in this study. Experiments were performed in closed, batch photo-bioreactors on a laboratory scale by using 500ml borosilicate Erlenmeyer flasks, securely plugged with non-absorbent cotton to allow aeration into the flask. The cultures were maintained in aseptic conditions, shaken periodically and placed under constant illumination.

4.5.1. Setup for wastewater treatment experiments:- For the study of wastewater remedial potential of *Chlorella vulgaris*, two experimental setups were maintained at the same time and under the same conditions. The first setup (Fig.4.2a) was of four borosilicate Erlenmeyer flasks of 500ml capacity each. Each of the flasks contained 245ml of the wastewater collected from Hulimavu Lake, supplemented with 5ml of *Chlorella* inoculum. The second setup (Fig. 4.2b) of four flasks of 500ml capacity flasks also contained 245ml of the wastewater sample in each flask, but without any algal inoculum. Both the experimental setups were incubated for a period of 15 days, from the time of inoculation.



(a) **(b)**

Figure 4.2: Experimental setup for wastewater treatment analysis (a) Incubation without *Chlorella* inoculum (b) Incubation with *Chlorella* inoculum

4.5.2. Setup for anti-bacterial experiment:- For investigation of anti-bacterial properties of *C. vulgaris*, the two Gram-negative, rod shaped, coliform bacterial strains *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* were used.

The parent cultures were obtained from the Microbiology Department of Mount Carmel College, Autonomous, Bengaluru. *E. coli* culture was maintained on MacConkey Agar medium (Table 4.2) while, *Klebsiella pneumoniae* was cultured on EMB Agar (Table 4.3) once in 15 days.

CONSTITUENT	CHEMICAL FORMULA	QUANTITY (per 100ml) pH= 7.1±0.2
Peptone (Pancreatic digest of gelatine)		1.7gms
Proteose peptone (Meat and Casein)		0.3gms
Lactose monohydrate	$C_{12}H_{24}O_{12}$	1gms
Bile salts		0.15gms
Sodium chloride	$NaCl$	0.5gms
Neutral Red		0.003gms
Crystal Violet		0.001gms
Agar	$C_{14}H_{24}O_9$	1.35gms

Table 4.2: Composition of MacConkey Agar Medium

CONSTITUENT	CHEMICAL FORMULA	QUANTITY (per 100ml) pH= 6.8±0.2
Peptone		1.0gms
Lactose	$C_{12}H_{22}O_{11}$	1.0gms
diPotassium hydrogen phosphate	K_2HPO_4	0.2gms
Eosin Y	$C_{20}H_6Br_4Na_2O_5$	0.004gms
Methylene Blue	$C_{16}H_{18}ClN_3S$	0.0065gms
Agar	$C_{14}H_{24}O_9$	1.5gms

Table 4.3: Composition of Eosin Methylene Blue (EMB) Agar Medium.

4.5.3. Setup for immobilization of algal strain:- A 2.5% solution of sodium alginate was used as the gelling agent and 2% calcium chloride solution was used as the stabilizing agent for the preparation of a matrix to immobilize the cells of *C. vulgaris*.

Methodology

5.1. Physical evaluation of the waste water quality

The quality of wastewater undergoing phycoremediation during the course of study was essentially studied for its physical parameters: colour, odour, pH and turbidity. These parameters were studied for both the experimental setups- without and with *Chlorella* inoculum. The colour and odour of the wastewater was determined simply through sensory perception and its turbidity was estimated using a turbidometer on the first day of the experimental setup and then again, on the 15th day from the day of setup. The pH of wastewater sample was analysed periodically, for both the setups, during the course of study (Table 5.1).

S.No.	Parameter	Method of Estimation
1.	Colour	Sensory perception of sight
2.	Odour	Sensory perception of smell
3.	pH	pH meter
4.	Turbidity	Turbidometer
6.	Dissolved Oxygen	Titartion (Winkler, 1888)
6.	Phosphate	Colorimetry (Strickland and Parsons, 1968)
7.	Nitrate	Atomic Spectrophotometry
8.	Chloride	Titration (Karl Friedrich Mohr, 1856)
9.	Carbonate	Titration
10.	Sulphate	Atomic Spectrophotometry

Table 5.1: Methodology for estimation of physical and inorganic parameters of wastewater

5.2. Preliminary estimation of inorganic nutrient content in the wastewater

Furthermore, the wastewater was checked for its nutrient concentration in terms of dissolved phosphate, nitrate, chloride, carbonate and sulphate content; prior to subjecting the wastewater sample to phycoremediation (Table 5.1). Estimation of phosphate was conducted using a colorimeter, titrimetric method was used to evaluate the chloride and carbonate content in the sample, while spectrophotometric technique was employed to determine the nitrate and sulphate content of the wastewater rendered from Hulimavu Lake. All chemicals and reagents used during the study were of analytical grade and, results of all estimations were recorded in triplicates.

5.2.1. Estimation of phosphate: Quantification of phosphorous (P) has been determined in terms of dissolved orthophosphate (H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}) and the determination is based on the method proposed by Strickland and Parsons in 1968 [60]. In this method, 4% ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] and antimony potassium tartrate [$K(SbO)C_4H_4O_6.1/2H_2O$] react in an acidic medium (5N H_2SO_4) with the orthophosphates present in the water sample to form a hetero-polyacid, antimony-phospho-molybdate complex which is then reduced by ascorbic acid to an intensely blue coloured complex. The absorbance of this complex is measured at 880nm wavelength.

5.2.2. Estimation of nitrate: The wastewater sample was sent to KSPCB for atomic-spectrometric determination of nitrate content in the sample.

5.2.3. Estimation of chloride: Quantification of chloride (Cl^-) in the wastewater sample has been determined using Mohr's titration method [61] (Karl Friedrich Mohr, 1856). In this method, the wastewater sample was titrated against 0.01N silver nitrate solution; standardized against standard chloride solution prepared from sodium chloride (NaCl). During the titration, chloride ions present in the wastewater are precipitated as white silver chloride (Eq. 1a) which is visualised as a reddish-brown precipitate using 5% potassium chromate (K_2CrO_4) as the end-point indicator (Eq. 1b). This stage is taken as evidence that all chloride ions have been precipitated.

$$\mathbf{Cl^- + AgNO_3 \longrightarrow AgCl\downarrow + NO^{3-}} \quad \textbf{(Equation: 1a)}$$

(White)

$$\mathbf{K_2CrO_4 + 2\,AgNO_3 \longrightarrow Ag_2CrO_4\downarrow + 2\,KNO_3} \quad \textbf{(Equation 1b)}$$

(Reddish brown)

Once a constant reading is achieved, the chloride content in the water sample is calculated using the given formula:

$$\mathbf{Cl^-\ (mg/ml) = \frac{\text{Titre value X Normality of } AgNO_3 \text{ X Gram equivalent wt. of chloride X 1000}}{\text{Volume of water sample}}}$$

5.2.4. Estimation of carbonate and bicarbonate: Titration of a definite quantity of wastewater sample against a standard acid viz. 0.02N sulphuric acid (H_2SO_4), using phenolphthalein and methyl orange as indicators, estimates the quantity of carbonates and bicarbonates present in the wastewater respectively [61]. Firstly, on adding the standard acid drop-wise, all the carbonate present in the sample is converted to bicarbonates (Eq. 2a). This is noted through the disappearance of the pink colour of phenolphthalein indicator. At this point, the carbonate content in the water sample was estimated using the given formula:

$$\mathbf{CO_3^{2-}\ (mg/ml) = \frac{\text{2XTitre value X Normality of sulphuric acid X Eq wt.of } CO_3^{2-} \text{ X 1000}}{\text{Volume of water sample}}}$$

$$2Na_2CO_3 + H_2SO_4 \xrightarrow{\text{Phenolphthalein (Pink)}} 2\,NaHCO_3 + Na_2SO_4$$

(Equation 2a)

(Colourless)

At this stage, methyl orange is added as the second indicator to the sample and continued titration against the same standard acid to note the colour change to orange, which marks the end-point (Eq. 2b).

$$2NaHCO_3 + H_2SO_4 \xrightarrow{\text{Methyl orange (colourless)}} Na_2SO_4 + H_2O + XO_2$$

(Equation 2b)

(Straw yellow)

The bicarbonate content in the water sample was calculated using the formula given below:

$$HCO_3^{2-}\ (\text{mg/ml}) = \frac{TV_M - TV_P \times \text{Normality of sulphuric acid} \times \text{Eq wt.of } HCO_3^{2-} \times 1000}{\text{Volume of water sample}}$$

where, TV_M is titre value obtained with methyl orange and,

TV_P is the titre value obtained with phenolphthalein.

5.2.5. Estimation of sulphate: The wastewater sample was sent to KSPCB for atomic-spectrometric determination of the sulphate content in the sample.

5.3. Determination of growth dynamics of *Chlorella vulgaris* in the wastewater

With respect to the primary objective of the study, which was to demonstrate the potential of *Chlorella vulgaris* in the treatment of wastewater, studying the growth kinetics of microalgal strain in the wastewater was essentially required. The sustenance and growth of the inoculated microalga in the experimental setup II (discussed in chapter 4) was recorded in terms of the total chlorophyll content present in the wastewater sample.

The chlorophylls are essential components of photosynthesis and can be denoted as the ‘life’ of green plants. These occur in the chloroplast, bound loosely to proteins, as green pigments and are easily extractable by organic solvents such as methanol. Estimation of the total chlorophyll content in the wastewater sample was based on the method proposed by McKinney in 1941[62]. A definite volume of the sample would be centrifuged at 13,400rpm for 10mins, and the resulting pellet was fetched in 1.5ml of 99.9% methanol to dissolve the chlorophyll in it. The optical density of the extract was measured at 652nm and 665nm wavelengths using spectrophotometer because at these wavelengths, maximum absorption of chlorophyll-a and chlorophyll-b takes place respectively. Using the absorption coefficients, the amount of chlorophyll is calculated as follows:

$$\textbf{Total Chlorophyll } (\mu g/ml) = 2.55 \times 10^{-2}\, A650 + 0.4 \times 10^{-2}\, A665.$$

5.4. Dissolved Oxygen content of wastewater

All living organisms are dependent upon oxygen in one form or another to maintain metabolic processes that produce energy for survival. The amount of oxygen dissolved in water is called the dissolved oxygen (DO) concentration and is influenced by several factors such as, the photosynthesizing organisms and water temperature. Algae and water plants liberate oxygen as a by-product of photosynthesis. The low solubility of dissolved oxygen is a major factor that limits the purification capacity of natural waters and necessitates the treatment of wastewater before discharge to the receiving water bodies.

Therefore, on estimating the amount of DO content in the wastewater sample, without and with inoculation of *Chlorella vulgaris*, would determine any difference in the oxygen content of wastewater brought about by the inoculated microalgal strain during the process of photosynthesis. The method that was followed for the determination of DO content in the water sample was based on the one proposed by Winkler in 1888[61]. Dissolved (or free) molecular oxygen present in water was estimated using an oxygen carrier [$Mn(OH)_2$] produced by the action of manganous sulphate [$Mn(SO_4)$] and potassium hydroxide (KOH) (Eq. 3a). $Mn(OH)_2$ so obtained reacts with the dissolved molecular oxygen in the wastewater sample to form a brown precipitate of basic manganic oxide [$MnO(OH)_2$] (Eq. 3b). $MnO(OH)_2$ then reacts with concentrated sulphuric acid to liberate nascent oxygen (Eq. 3c), that oxidizes potassium iodide (KI) to iodine (I_2) (Eq. 3d). This liberated iodine was then titrated against 0.025N sodium thiosulphate solution using 1% starch as an indicator [11].

$$\mathbf{2NaOH + MnSO_4 \longrightarrow Mn(OH)_2 + K_2SO_4} \qquad \textbf{(Equation 3a)}$$

$$\mathbf{2Mn(OH)_2 + O_2 \longrightarrow 2MnO(OH)_2\downarrow} \qquad \textbf{(Equation 3b)}$$

(brown)

$$\mathbf{MnO(OH)_2 + H2SO4 \longrightarrow MnSO_4 + 2H_2O + [O]} \qquad \textbf{(Equation 3c)}$$

$$\mathbf{2KI + H_2SO_4 + [O] \longrightarrow K_2SO_4 + H_2O + I_2} \qquad \textbf{(Equation 3d)}$$

Dissolved oxygen level of the wastewater sample was calculated using the given formula [61]:

DO(mg/l) = <u>Titre value X Normality of sodium thiosulphate X Equivalent wt. of oxygen</u>

Volume of the sample X 100

5.5. Determination of the anti-bacterial potential of *Chlorella vulgaris*

In order to remediate the wastewater at a biological level as well, the potential of *Chlorella vulgaris* as an anti-bacterial agent was evaluated against two bacterial strains namely, *Escherichia coli* and *Klebsiella pneumoniae*. These pathogenic strains of bacteria are infamous for carrying several water borne diseases such as diarrhoea, dysentery and pneumonia; and also cause abdominal pain and gastroenteritis at an acute level.

To determine the anti-bacterial effect of *Chlorella vulgaris* against the selected Gram-negative bacterial strains, the standard well-diffusion technique was adopted. The anti-bacterial potential of the microalgal strain was studied at three different concentrations of 0.1μg/ml, 0.5μg/ml and 0.9μg/ml. Similar procedure was followed for both the bacterial strains *E. Coli* and *K. pneumoniae,* wherein the bacterial strain was fed on Mueller-Hinton Agar medium (Table 5.2) by streak-plate method. *Chlorella vulgaris* was then fed to the plate, in wells 5mm deep to act upon the bacteria by the well-diffusion method. Only one algal concentration at a time was used in the agar plate and tetracycline was used as the ‘control’. The plates were incubated for 18 hours at 37°C after which, the zone of inhibition was read.

S.No.	Ingredients	Quantity (grams per Litre)
1	Beef extract	2gms/l
2	Acid Hydrolysate of casein	17.5gms/l
3	Starch	1.5gms/l
4	Agar	17gms/l

Table 5.2: Composition of MH Agar medium [63]:

5.6. Immobilization of *Chlorella vulgaris* in a matrix of sodium alginate

In order to accomplish the secondary objective of the study i.e. solve the problem of collection of proliferating algal biomass from wastewater treatment plants, a strategy was worked upon to trap the phyco-remediating algal strain within a matrix such that its growth area is limited and so, harvesting method be easy. Therefore, for this study, *Chlorella vulgaris* was immobilized in a matrix of 2.5% sodium alginate ($C_6H_9NaO_7$). The immobilization technique was as such that, an equal volume of *Chlorella vulagris* culture and 2.5% sodium alginate was taken and drop-wise added to 2% calcium chloride solution ($CaCl_2$). In presence of calcium, viscous solution of sodium alginate forms an enclosed structure binding the algal strain within the matrix (Fig. 5.1). Such 'algal beads' not only hold substantial cell density but are also easy to work with.

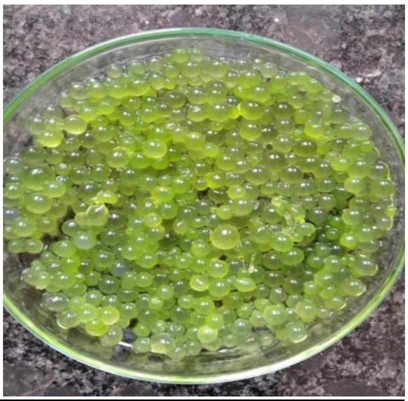


Figure 5.1: Sodium alginate beads of *Chlorella vulgaris.*

Results

6.1. Physical evaluation of the waste water quality

***6.1.1. Colour**:* The colour of Hulimavu Lake water at the time of collection was perceived as greenish and murky. The colouration can be attributed to the presence of potentially toxic organic compounds like fulvic acids, proteins and lignin liberated as degeneration products of fishes and macrophytes covering the surface water (Fig. 6.1a) and inorganic elements such as sulphates and carbonates released through synthetic detergents discharged as municipal wastewater from the neighbouring settlements [14](Fig. 6.2b). However, the colour of wastewater during its treatment with microalga *Chlorella vulgaris* remained unchanged because of the growing green algae imparting the green colouration to wastewater.

6.1.2. Odour: Hulimavu Lake water had a faint, foul smell at the time of sampling of water. This is primarily due to the odour liberated from the domestic waste disposed in and around the lake area (Fig. 6b), contaminating the water with effluents rich in nitrates, phosphates and sulphates [14]. On treatment with *Chlorella vulgaris*, the odour of the wastewater smell was perceived as a 'fishy', 'rotten egg' smell; probably because of the bio-oxidation of organic matter by micro-organisms, and maybe because of any bioactive compounds such as, alkaloids released by *Chlorella* into the medium.

(a)

(b)

Figure 6.1: Site of collection of wastewater from Hulimavu Lake (a)Macrophyte cover over the lake, (b) Waste disposal around the lake.

***6.1.3. Turbidity**:* Turbidity is a measure of the relative clarity of a suspension. It is caused by the suspended particulate matter that absorbs the sun rays and scatters the light making the water appear hazy and cloudy. The extent of haziness was measured in terms of Nephelometric Turbidity Units (NTU) using a turbidometer. During the course of study, the turbidity of the wastewater was increased by 2.4 units after inoculation with *Chlorella vulgaris* (Table 6.1). This is because of the growing microalgae which restrict penetration of light into the medium (Fig. 6.2).

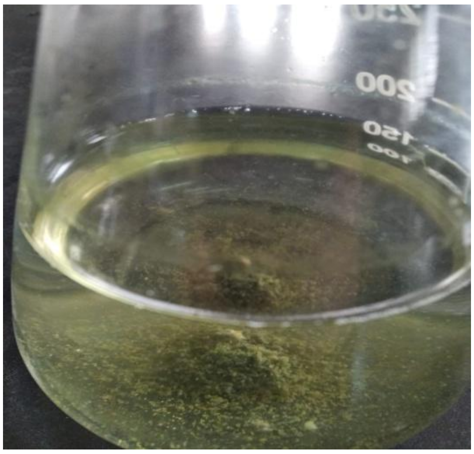


Figure 6.2: Colour and turbidity of wastewater sample (without inoculation) as collected from Hulimavu Lake

S. No.	Parameter	Without Chlorella inoculum	With Chlorella inoculum
1.	Colour	Olive green	Dark, olive green
2.	Odour	Putrid	Fresh algal smell
3.	Turbidity	11.1NTU	13.5NTU

Table 6.1: Physical analysis of the wastewater

***6.1.4. pH**:* pH can be defined as the negative logarithm of the hydrogen-ion concentration in the sample, that determines its intensity of acidity (below pH = 7) or alkalinity (above pH = 7). The pH scale is usually represented as ranging from 0 to 14, with pH 7 at 25°C representing absolute neutrality. The pH value governs the solvent properties of water and determines the extent and type of physical, chemical and biological reactions likely to occur within the water system. During the course of study, the pH of wastewater was determined periodically for both the experimental setups – without and with *Chlorella* inoculum (Table 6.2a and 6.2b), to study the effect of *Chlorella vulgaris* on the acidity/alkalinity of the wastewater. The results obtained clearly show a shift in the pH of wastewater towards alkalinity post treatment with *Chlorella* spp.

S. No.	Day of Reading	pH = -log [H+]	
		Hydrogen-ion conc.(moles/l)	pH
1.	Zero-th Day (Initial)	8.5×10^{-8}	7.07
2.	Third day	7.9×10^{-8}	7.10
3.	Ninth Day	6.4×10^{-8}	7.19
4.	Fifteenth Day (Final)	6.3×10^{-8}	7.20

Table 6.2a: Estimation of pH of wastewater without inoculation

S. No.	Day of Reading	pH = -log [H+]	
		Hydrogen-ion conc.(moles/l)	pH
1.	Zero-th Day (Initial)	8.5×10^{-8}	7.07
2.	Third day	6.4×10^{-8}	7.19
3.	Ninth Day	2.6×10^{-8}	7.58
4.	Fifteenth Day (Final)	8.91×10^{-8}	8.05

Table 6.2b: Estimation of pH of wastewater after inoculation

6.2. Growth dynamics of *Chlorella vulgaris* in the wastewater

As discussed earlier in Chapter 2, wastewater essentially contains all nutrients required for microalgal growth such as, carbon source, nitrates and phosphates. These organic and inorganic nutrients nourish the inoculated microalgal strain *Chlorella vulgaris* to grow in the water system and potentially remediate the wastewater by absorbing the nutrients for energy in return of oxygen necessary for other life forms. The growth pattern of *Chlorella vulgaris* in the wastewater was obtained through an estimation of the total chlorophyll content in the wastewater, during the bioprocess time of 15 days (Table 6.3b). For the purpose of comparison, chlorophyll content of the wastewater without inoculation with Chlorella, was also determined (Table 6.3a)

S.No.	Day of reading	A(652)	A(665)	Chl-a (µg/ml)	Chl-b (µg/ml)	Total Chlorophyll (µg/ml)
1	Zero'th (Initial)	0.02	0.0156	0.4139	0.3589	0.7728±0.879
2	Third	0.025	0.033	0.7355	0.6144	1.3499±0.671
3	Ninth	0.02	0.035	0.727	0.472	1.199±0.842
4	Fifteenth (Final)	0.0333	0.0859	1.6574	0.7192	2.3766±0.963

Table 6.3a: Estimation of chlorophyll content in wastewater without inoculation

S.No.	Day of reading	A(652)	A(665)	Chl-a	Chl-b	Total

				(µg/ml)	**(µg/ml)**	**Chlorophyll (µg/ml)**
1	Zero'th (Initial)	0.0352	0.0385	0.907	0.8769	1.7839±0.942
2	Third	0.04	0.044	1.0347	1.002	2.0367±1.001
3	Ninth	0.0398	0.0727	1.4968	0.9125	2.4093±0.965
4	Fifteenth(Final)	0.0433	0.0917	1.8321	0.981	2.8131±0.984

Table 6.3b: Estimation of chlorophyll content in wastewater with inoculation

6.3. Estimation of inorganic nutrient content in the wastewater

As discussed earlier in Chapter 2, wastewater essentially contains all of the nutrients required by microalgae to continue their growth in the medium. Similarly, during the course of study, *Chlorella vulgaris* was expected to consume the inorganic nutrients present in the wastewater for its growth and proliferation; in turn remediating the wastewater by lowering the dissolved inorganic substances such as, phosphates (Table 6.4), chlorides (Table 6.5), carbonate and bicarbonate (Table 6.6a and 6.6b), nitrate and sulphate (Table 6.7) from toxic levels to less or non-toxic values. To note the potential effect of *Chlorella vulgaris* in remediating the wastewater, the inorganic nutrient concentration in the water sample was estimated before and after inoculation, during a bioprocess time of 15 days.

6.3.1. Estimation of phosphate: Results obtained after treating the wastewater with *Chlorella vulgaris* for a period of 15 days clearly reveal a 15% reduction in the phosphate content of the water.

S. No.	**Day of Reading**	**Sample without inoculation**		**Sample with inoculation**	
		A (880)	**Phosphate level (µg/ml)**	**A (880)**	**Phosphate level (µg/ml)**
1.	Zero-th Day (Initial)	1.52	42±0.3756	1.51	42±0.3756
2.	Third day	1.49	41±0.0577	1.38	39±0.0333
3.	Fifteenth Day (Final)	1.60	45±0.0416	0.92	26±0.5487

Table 6.4: Estimation of phosphate content in wastewater

6.3.2. Estimation of chloride: Treatment of wastewater with *Chlorella vulgaris* showed a 28% reduction in the chloride content of the wastewater within 15 days.

S. No.	**Day of Reading**	**Sample without inoculation**		**Sample with inoculation**	
		Titre value (ml)	**Chloride level (mg/l)**	**Titre value (ml)**	**Chloride level (mg/l)**
1.	Zeroth Day (Initial)	20.0	1949.0 ± 0.235	20.0	1949.0±0.456
2.	Third day	24.60	1958.61±0.357	22.0	1719.325±0.532
3.	Ninth day	19.40	1506.62±0.419	10.2	682.412±0.238
3.	Fifteenth Day (Final)	19.0	1471.175±0.862	8.9	567.20±0.986

Table 6.5: Estimation of chloride content in wastewater

6.3.3. Estimation of carbonates and bicarbonates: Remediation of wastewater with *Chlorella vulgaris* was further substantiated with 11.11% reduction in the carbonate content and 10.86% reduction in the bicarbonate content of the wastewater, within 15 days.

S. No.	Day of Reading	Sample without inoculation		Sample with inoculation	
		Titre value (ml)	Carbonate level (mg/l)	Titre value (ml)	Carbonate level (mg/l)
1.	Zero-th Day (Initial)	0.70	42.0±0.259	0.8	48.0±0.247
2.	Third day	0.80	48.0±0.601	0.8	48.0±0.658
3.	Ninth day	1.0	60.0±0.590	0.6	36.0±0.512
4.	Fifteenth Day (Final)	1.20	72.0±0.487	0.5	30.0±0.637

Table 6.6a: Estimation of carbonate content in wastewater

S. No.	Day of Reading	Sample without inoculation		Sample with inoculation	
		Titre value (ml)	Bicarbonate level (mg/l)	Titre value (ml)	Bicarbonate level (mg/l)
1.	Zero-th Day (Initial)	3.8	93.0±0.842	3.8	90.0±0.820
2.	Third day	4.1	99.0±0.345	3.2	72.0±0.756
3.	Ninth day	4.0	90.0±0.853	2.4	54.0±0.349
4.	Fifteenth Day (Final)	4.3	93.0±0.851	2.5	60.0±0.800

Table 6.6b: Estimation of bicarbonate content in wastewater

6.3.4. Estimation of nitrate and sulphate: The nitrate level in wastewater was reduced by 12.72% post treatment with *Chlorella vulgaris* after 15 days. However, a slight increase in the sulphate content of the wastewater was noted.

S. No.	Parameter	Sample without inoculation (Initital Reading)	Sample with inoculation (Final Reading)
1.	Nitrate (mg/l)	15.5	12.0
2.	Sulphate (mg/l)	38.0	39.0

Table 6.7: Estimation of nitrate and sulphate content in wastewater

6.4. Estimation of dissolved oxygen content in the wastewater

Stabilization of organic and inorganic matter when discharged untreated or partially treated in receiving waters, leads to depletion in the dissolved oxygen content of the water and harms the overall ecology of the aquatic system. With regards to the objective of phyco-remediating such wastewaters with the help of *Chlorella vulgaris*, which on photosynthesis should liberate oxygen into the water system, the following results were obtained as mentioned in table 6.8. Phyco-remediation of the wastewater with *Chlorella vulgaris* caused a 18.2% increase in the oxygen content of the wastewater within 15 days.

S. No.	Day of Reading	Sample without inoculation		Sample with inoculation	
		Titre value (ml)	DO level(mg/l)	Titre value (ml)	DO level (mg/l)
1.	Zero-th Day (Initial)	9.1	26.76	9.1	26.76
2.	Third day	10.2	28.192	11.5	28.192
3.	Fifteenth Day (Final)	10.5	28.874	15.5	44.95

Table 6.8: Estimation of DO content in wastewater

6.5. Determination of the anti-bacterial potential of *Chlorella vulgaris*:

The following results were obtained (Table 6.9) after testing the activity of *Chlorella vulgaris* against the bacterial strains *Escherichia coli* and *Klebsiella pneumoniae*, wherein the microalgal strain showed the best anti-bacterial against *E. coli* at a conc. range of 0.5 – 0.9µg/ml (Plate.6.1a) and against *K. pneumoniae* at a conc. of 0.9µg/ml (Plate. 6.1b). The results so obtained also show the anti-bacterial effect of *C. vulgaris* against *E. coli* almost similar to streptomycine used as the control, in the conc. range of 0.5 – 0.9µg/ml.

S. No.	Microalgal concentration in µg/ml (Solvent – Ethanol)	*Escherichia coli*	*Klebsiella pneumoniae*	Control (Streptomycine)
1.	0.1	12mm ±0.1	6mm±0.14	15mm±0.2
2.	0.5	21mm±0.12	11mm±0.2	19mm±0.2
3.	0.9	27mm±0.09	18mm±0.13	26mm±0.1

Table 6.9: Determination of the zone of inhibition by *Chloella vulgaris* against *E. coli* and *K. pneumoniae.*

(a) **(b)**

Plate 6.1: Zone of inhibition obtained with *Chlorella vulgaris* (a) against *E. coli* at 0.5µg/ml, (b) against *K. pneumoniae* at 0.9µg/ml

6.6. Estimation of phosphate content in wastewater with immobilized cell of *Chlorella vulgaris*:

Treatment of wastewater with immobilized microalgal strain *C. vulgaris* showed faster remedial tendency against the conventional suspension culture, in reducing the phosphate levels of wastewater by 27% in a span of 3 days (Table 6.10).

S. No.	Day of Reading	Sample without inoculation		Sample with inoculation	
		A (880)	Phosphate level (µg/ml)	A (880)	Phosphate level (µg/ml)
1.	Zero-th Day (Initial)	1.164	46.56±0.159	0.9401	37.404±0.19
2.	First day	1.152	46.51±0.268	0.6584	25.167±0.537
3.	Second Day (Final)	1.178	47.11±0.467	0.4119	16.476±0.421

Table 6.10: Estimation of phosphate content in wastewater using immobilized algae

During the course of study, it was also observed that on treating the wastewater with immobilized *C. vulgaris* beads,

1. the integrity of the beads gradually changed and reduced in size as it absorbed the nutrients.
2. the turbidity of the wastewater was not increased, as perceived with the use of suspension microalgal culture.

Discussion

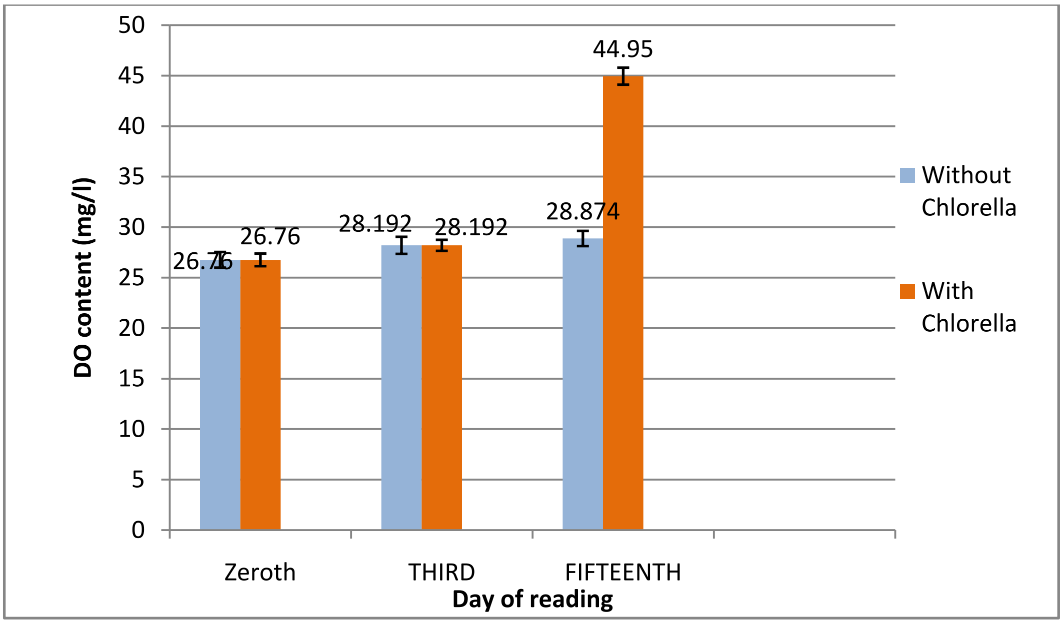
Untreated and unmanaged wastewater discharged from various domestic, agricultural and industrial activities can become a store of organic and inorganic substances which increase to toxic levels that can harm the overall aquatic ecosystem. Not only is the physical and chemical composition of the water system misbalanced by the influx of these substances; but also does it cause a large number of fatal water-borne diseases to the humans and cattle consuming such infected waters. Treatment of this wastewater is a matter of hydrological and ecological concern that is looked upon by various researchers. In this study, microalgae based remediation of wastewater, rendered from Hulimavu Lake, laden with inorganic nutrients, has been given prime importance.

7.1. Algae cell growth

The microalgal strain *Chlorella vulgaris* can be cultured by different methods and under a range of conditions while, all it needs is light as an energy source to convert the absorbed water and carbon dioxide into biomass through photosynthesis [14]. Therefore, for this study, the necessary conditions were provided to achieve exponential growth of the microalgal strain in the wastewater. Photosynthetic products accumulate in various forms such as, cell components or storage materials that assist in the growth of the algal species in the water medium [4]. According to Beer's Law, the amount of light that passes through the algae suspension will be inversely proportional to the algae concentration; which was well perceived during the preliminary examination of the wastewater. Wherein, turbidity of the wastewater increased by 2.4 units post inoculation with *C. vulgaris*.

Wastewater is a good source for cultivation of microalgae as it is rich in all the macro- and micro-nutrients essential for growth of algae. The dynamics of microalgal growth in the wastewater sample, over the period of 15 days, was checked in reference to the total chlorophyll content in the medium. The growth of *Chlorella vulgaris* was noted with an increase in the chlorophyll concentration from the time of inoculation to the 15th day of the study span.

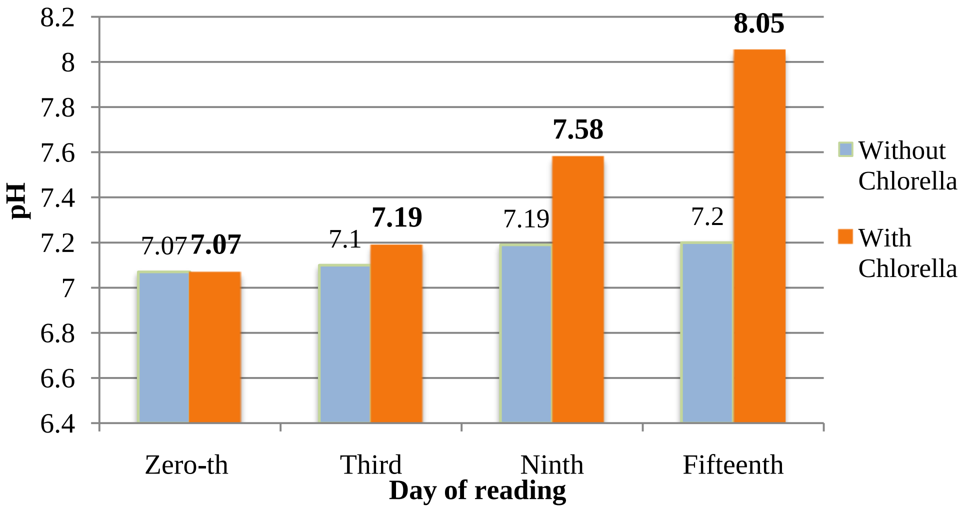
According to our results, *Chlorella vulgaris* fixing CO_2 from wastewater i.e. photosynthesising, indicated that this organism could be suggested as a potent microalgae for CO_2 sequestration, as first demonstrated by Oswald and Gotaas in 1955 [1]. Under waste water stabilization conditions, the algae produced high level of oxygen (18.2%) as a by-product of photosynthesis. This oxygen was utilised by the bacteria to bio-oxidise the organic compounds present in the wastewater (Fig. 7.1), as reported by Zimmo *et al.* in 2000 [22] while working on algae based waste stabilization ponds. It is reported that the batch culture of *C. vulgaris* grown in wastewater reached high chlorophyll-a concentration of 1.8321µg/ml on the 15th day of our study. This increase in CO_2sequestration is very efficient in manuevering the oxygen content in the aquatic ecosystem.



Graph 7.1: Estimation of DO content in wastewater

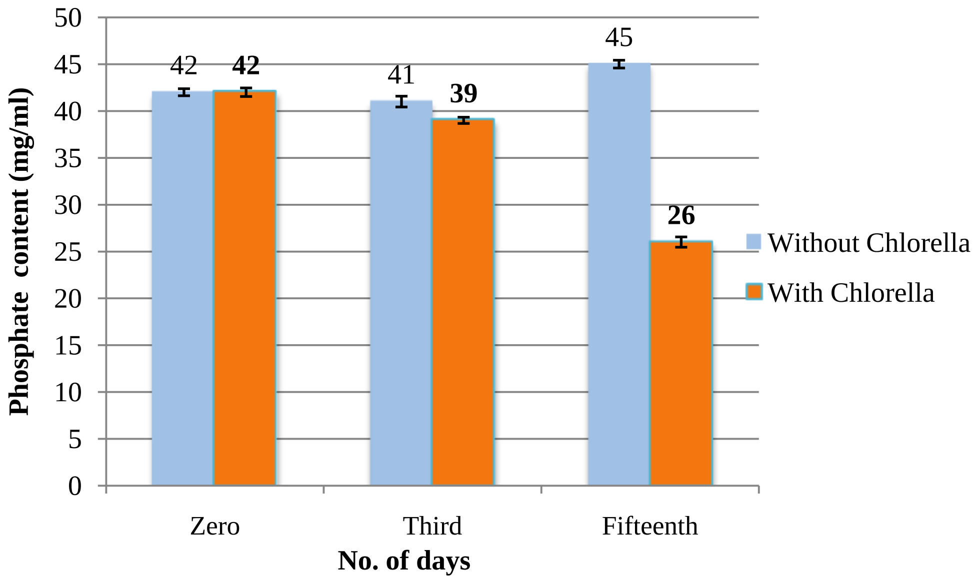
7.2. Remediation of inorganic nutrients from wastewater

During the process of phyco-remediating the wastewater, pH levels increased initially and thereafter remained around 8.0. The microalga *Chlorella vulgaris* reduces dissolved CO_2 concentrations through photosynthesis which, in turn, raises the pH level (Graph 7.2). Becker E.W. (1994) [64] illustrated that such pH levels are maintained so that there shall be no increase in ammonia concentrations; because a shift in pH level above 9 would raise ammonia concentration. Ammonia is extremely toxic where it not only contributes to the unpleasant odour of the water body but also depletes the oxygen level in the water. Hence, pH stabilisation during microalgal treatment of wastewater not only keeps the ammonia levels in check but also influences the metabolic process by which the bacteria would decompose the dissolved organic matter [64].



Graph 7.2: pH level of wastewater during phycoremediation process

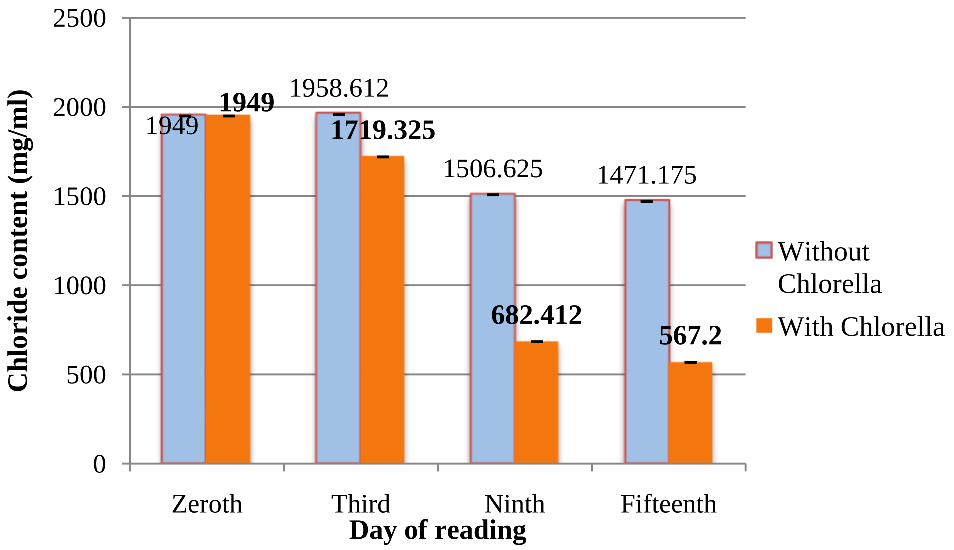
7.2.1. Phosphorous removal: Phosphate removal upto 15% by *C. vulgaris* during phycoremediation study is due to the utilisation of phosphorous (P) as a macro-nutrient for growth (Graph 7.3). The phosphorous, is used in algal cells mainly for production of phospholipids, adenosine triphosphates (ATP) and nucleic acids, and gets assimilated as inorganic orthophosphates [65]. Thus, the phosphates from wastewater were removed by photosynthetic assimilation and calcium phosphate precipitation because of high pH levels caused by high algal photosynthetic activity [16]. Therefore, chemical stripping of phosphorous can be regarded as an advantageous consequence of the algal growth.



Graph 7.3: Decrease in phosphate level of wastewater during phycoremediation process

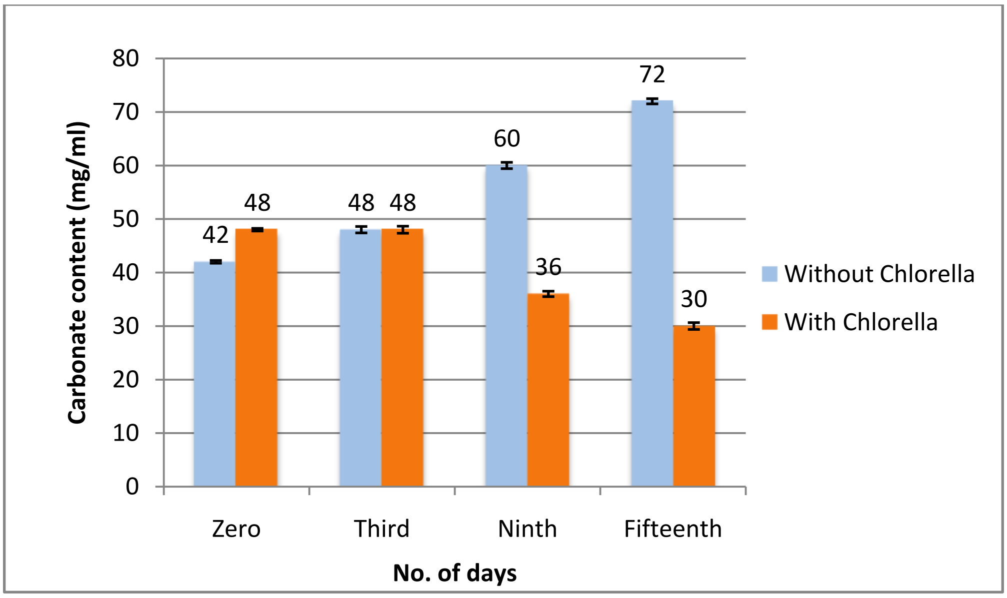
7.2.2. Nitrogen removal: Nitrogen is another crucial parameter and is becoming increasingly essential in wastewater management because presence of nitrogen has adverse ecological impacts as reported in [17] and can affect public health. Although nitrate (NO_3^-) itself is not toxic, its conversion to nitrite (NO_2^-) is potentially fatal. In the body, nitrite can oxidise iron (II) and form methaemoglobin, which binds to oxygen less effectively than normal haemoglobin [11]. All forms of nitrogen are absorbed as nutrients by the microalga, although the most common nitrogen compounds assimilated by microalgae are ammonium (NH_4^+) and nitrate [65]. In our study, *C. vulgaris* was able to reduce all nitrate levels substantially, similar to the observations of [12], by 12.72% and ammonia concentrations were kept in check as well.

7.2.3. Chlorine removal: Chloride (Cl^-) is an essential micronutrient that is needed by microalgae exposed to saline conditions in regulation of cell turgor and volume [65]. In our study, *C. vulgaris* remediated 28% of the chlorine content of the wastewater for maintaining its membrane integrity under the saline conditions of the wastewater (Graph 7.4).

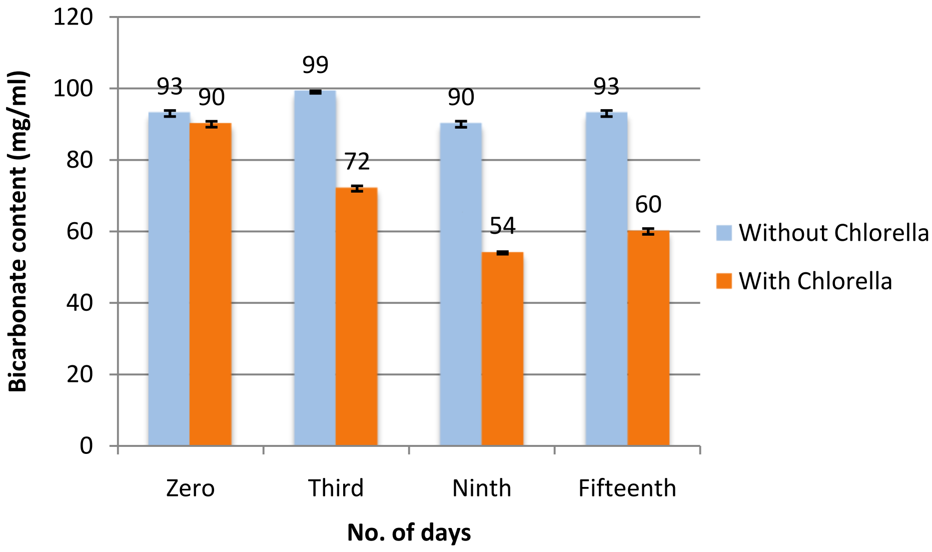


Graph 7.4: Decrease in chloride level of wastewater during phycoremediation process

7.2.4. Carbonate and bicarbonate removal: For similar reasons of photosynthesis and meeting energy requirements, *C. vulgaris* depends on dissolved carbonates and bicarbonates as C-source. Therefore, during its growth period of 15 days into wastewater medium, the carbonate levels dropped by 11.11% (Graph 7.5) and the bicarbonate level was dropped (Graph 6) by 10.86%. Furthermore, reduction in the carbonate and bicarbonate content of the wastewater by the microalgae is an attempt to reduce the hardness of water, which is necessary to improve the physical characteristics of the water system.



Graph 7.5: Decrease in carbonate level of wastewater during phycoremediation process



Graph 7.6: Decrease in bicarbonate level of wastewater during phycoremediation process

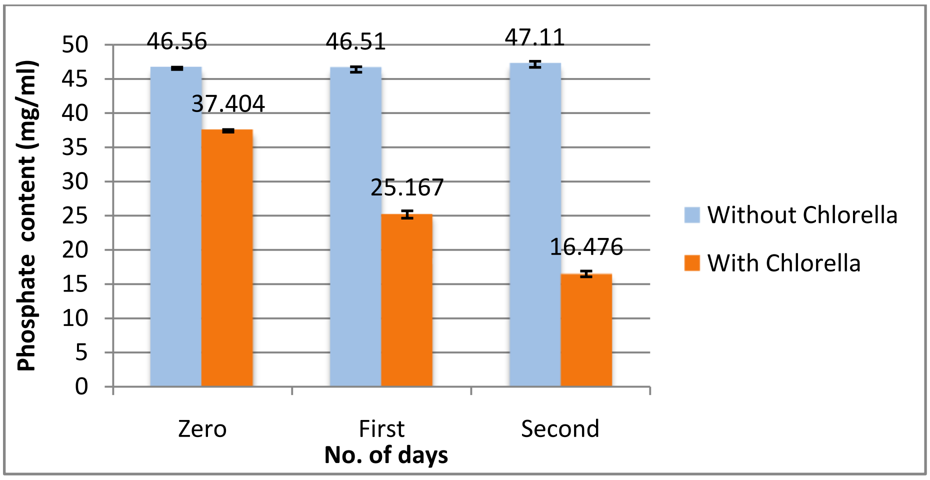
7.2.5. Sulphate removal: Two forms of sulphur are commonly found in water systems- sulphate (SO_4^{2-}) and hydrogen sulphide (H_2S). Both forms are a nuisance to the physical quality of the water body. Sulphur-reducing bacteria that usually flourish on the hot-water side of a distribution system consume the sulphates as an energy source and in turn liberate H_2S [15]. This could be a reason as to why the putrid, fishy-smell was perceived in the wastewater during our course of study. However, unlike the work of Pathak *et al* (2014) carried on remediating textile wastewater [15]; the present study did not accomplish in remediating the wastewater of its dissolved sulphate content, brought about by domestic activities in and around the lake area such as, washing clothes and bathing. This could be attributed to a balance that was maintained by the sulphur released (H_2S) by sulphur-reducing bacteria in order to control blooming of *C. vulgaris*. This is why, sulphate algicides are commonly used to treat algal blooms.

7.3. Remediation of wastewater at biological level

The anti-bacterial potential of *C. vulgaris* was well demonstrated during the study against two fecal coliform bacterial strains *Escherichia coli* and *Klebsiella pneumoniae* commonly found proliferating in wastewaters of such kind. *E. coli* is a Gram-negative rod-shaped bacteria; causative agent of abdominal pain, nausea, gastroenteritis and infectious diarrhoea in human beings. *K. pneumoniae* is also a Gram-negative rod-shaped bacteria; the primary causative agent of pneumonia that spreads through contaminated water. The ethanolic extract of *Chlorella vulgaris* showed an anti-bacterial activity against *E. coli* in a conc. range of 0.5-0.9µg/ml and worked against *K. pneumoniae* at a concentration of 0.9µg/ml. hence, the extract can be suggested to treat infections like those caused by *E. coli* and *K. pneumoniae.* Also, the anti-microbial activity of ethanolic extract of *C. vulgaris* worked against the two selected bacterial strains at the same level as the n-Hexane extract of *C. pyrenoidosa* worked on by [34].

7.4. Remediation of wastewater by immobilized algae

Remediation of wastewater using *C. vulgaris* was given a whole new perspective when the microalga was immobilized in beads of sodium alginate and calcium chloride. The results clearly show an efficient reduction in the phosphate level (Graph 7.7) of wastewater by 27% in just 3 days. This is because, once the alga is immobilized within the matrix, it needs to photosynthesise and grow within that encapsulated space only; thereby increasing cell density, consequently remediating the wastewater of its phosphorous content at a faster rate than the suspension culture of *C. vulgaris* used earlier in the study. These results function on the same fundamentals as obtained by [66] and would the bioprocess time of this study be longer, similar results could be achieved.



Graph 7.7: Decrease in phosphate level of wastewater during phycoremediation process using immobilized algal beads

Conclusion

With respect to the results obtained during this study focused on studying the potential of microalgal strain *Chlorella vulgaris* in remediating wastewater of its physical, chemical and biological properties, it can be concluded that *C. vulgaris* is a potent candidate for sustainable treatment of wastewater as a bio-remediator. *C. vulgaris* is a robust green microalga that withstands the toxic levels of phosphate, nitrate, chlorine, carbonate and sulphate present in the wastewater and shows steady increase in growth. During the exponential phase of growth, *C. vulgaris* cultivated in nutrient rich wastewater medium, tends to accumulate or absorb the dissolved inorganic nutrients for the purpose of growth, synthesis of stored organic compounds (proteins, lipids and carbohydrates) and need of energy. Therefore, compared to synthetic media, wastewater can be used as a cheap, efficient and sustainable method of cultivating green freshwater microalgal species such as *C. vulgaris.*

The primary objective of any remediation project is removing the toxic phosphates present in the water system, as it is the leading cause of eutrophication. *C. vulgaris* is an efficient cleanser of phosphates from wastewater and hence, proves to have a positive role in remediation of any kind of wastewater. *C. vulgaris* also removed 12.5% of the dissolved nitrates in the wastewater. Therefore, the microalgal strain should be absorbing nitrates to synthesise nucleic acids and proteins essential to maintain cell integrity. The absorption of chlorides from wastewater by *C. vulgaris* can be attributed as a need of the microalga for maintenance of membrane integrity and permeability to allow the absorption of other inorganic nutrients, essential for growth. As an added advantage of treating wastewater with microalgae is that, the microalgal strain consumes the dissolved carbonates as a source of carbon for photosynthesis. In the same way, *C. vulgaris* consumes dissolved carbonates and bicarbonates in the wastewater media, to be able to continue photosynthesising and consequently increasing the softness of the water. As a result of photosynthesis, *C. vulgaris* releases oxygen as a by-product that gets incorporated into the hydrological system, thereby rejuvenating the wastewater. An increase in the dissolved oxygen content of the water system is crucial to sustain life below the surface of water. Presence of sufficient amount of oxygen in the water body allows sustenance of life and harmony in the ecological balance of the water system. Furthermore, the chemical by-products of photosynthesis, make the water medium alkaline. This shift in alkalinity of wastewater is achieved through photosynthesising *C. vulgaris* and is necessary to curb the levels of ammonia in the water system below toxic levels. The alkalinity is also a mediating factor for the metabolic activities of bacteria that bio-oxidise the organic nutrients present in the wastewater.

At a biological level, the ethanolic extract *C. vulgaris* proved to be a potent anti-bacterial agent against *Escherichia coli* and *Klebsiella pneumoniae* commonly found in wastewater systems. These two bacterial strains are the prime causative agents of various water borne diseases such as diarrhoea, gastroenteritis and pneumonia in humans. Therefore, under the influence of otherwise difficult conditions such as salinity, as found in wastewaters, *C. vulgaris* is able to continue growth and synthesise organic compounds that resemble the potency of streptomycine against pathogenic bacterial strains *E. coli* and *K. pneumoniae.* In view of foregoing results, it is concluded that extracts of *C. vulgaris* have the potential to work as antimicrobial agents, which may be exploited either by application of individual extracts or in combination with chemical antibiotics. The anti-oxidative potential of such algal extracts would always contribute positively in pharmaceutical of the algal extracts.

An algal based wastewater treatment system (AWTS) is therefore a composite technique of remediating wastewater at physical, chemical and biological level. Post the bioprocess, this algal biomass can be collected and used for further processing. As the algae have substantially accumulated the dissolved nutrients from the wastewater system inside their cellular system; which can be extracted for commercial use eg. Lipids, carbohydrates etc.

The ease of harvesting the microalgae from such AWTS is obtained if the microalgal strain is encapsulated in a gel-like matrix. Therefore, *C. vulgaris* entrapped within a matrix of (2.5%) sodium alginate and (2%) calcium chloride proved to be easy to work with during the course of study and its collection of easier as compared to the suspension culture.Immobilized strain of *C. vulgaris* showed enhanced phosphorous removal (27.5%) from the wastewater within a span of just 3 days. Hence, the increased cell density when forming algal beads increases the remediation kinetics of the entrapped microalgae. Also, this matrix of sodium alginate and calcium chloride is permeable to the nutrients dissolved in the water system. The use of immobilized algal strains in remediation of wastewater does not cause turbidity of the medium as compared to the suspension culture, because if immobilized, the algal strain is spatially restricted and cannot cause cloudiness of the medium. Altogether, an immobilized algal based wastewater treatment is advised in accordance to the results obtained with this study.

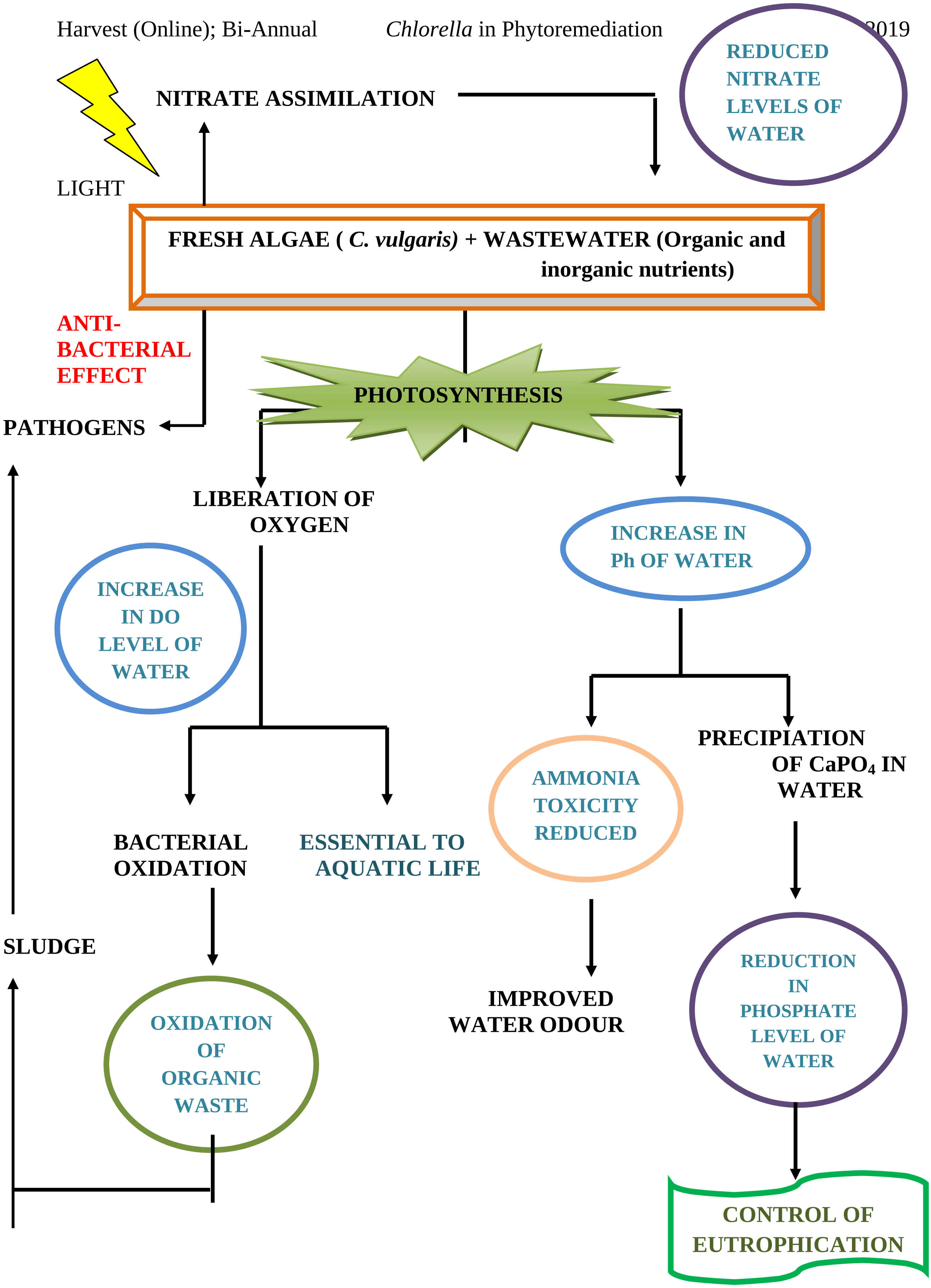


Figure 8.1: An outline of the remediation of Hulimavu wastewater by *C. vulgaris*

Future Prospectives

This study undertaken to analyse the wastewater remediation potential of microalgal strain *Chlorella vulgaris* and its anti-microbial potential against disease causing bacterial strains *Escherichia coli and Klebsiella pneumoniae* commonly found in wastewaters gives way to a variety of opportunities that can be looked upon, to work towards a sustainable future.

1. In accordance to the results obtained during the course of study, an algal based wastewater treatment system (AWTS) using *C. vulgaris* is advised in place of conventional wastewater treatment systems to achieve a composite treatment of wastewaters in terms of their physical, chemical and biological parameters.
2. A cost-effective AWTS could be designed wherein; water discharged from domestic, agricultural and industrial settlements is treated before disposal into water bodies. In this way, ecological system of water bodies, such as that of Hulimavu Lake, is not hampered.
3. Such AWTS could also be utilized to treat hospital residual wastes that pose to be great threat to human health as these wastes house a variety of pathogenic organisms that spread diseases.
4. *C. vulgaris* can be processed to function as a water purifier and in reducing the hardness of water for domestic use.
5. The technology of immobilization of algae can be taken into a higher level and employed in such AWTS for batch-wise remediation of contaminated waters.
6. The anti-bacterial compounds synthesized by *C. vulgaris* can be used in the pharmaceutical industry as well.
7. Post bio-remediation experiments, *C. vulgaris* can be harvested from the treatment plants and processed further for extraction of valuable compounds such as lipids and proteins, which can be utilized in the manufacture of biofuel and biofertilizers.
8. Biofilms of *C. vulgaris* around pebbles can be used to remediate a small scale water system such as an aquarium. This would also add to its aesthetic beauty.

References

1. Gotaas H. B and W. J. Oswald, (1955). Photosynthesis in sewage treatment. *J. Sanit. Engg Div. Am. Soc. Civ. Engrs*., 81: 686-893.
2. de-Bashan LE and Bashan Y, (2010). Immobilized microalgae for removing pollutants: Review of practical aspects. *Bioresour Technol*; 101(6): 1611–1627.
3. de-Bashan LE and Bashan Y, (2003). Recent advances in removing phosphorus from wastewater and its future use as fertilizer. *Water Res*; 38:4222-4246.
4. Tam N.F.Y. and Y.S. Wong (1989). Wastewater nutrient removal by Chlorella pyrenoidosa and Scenedesmus sp. *Environ. Pollut*., 58: 19-34.
5. Mallick, N. (2002). Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A review. *Biometals*. 15: 377-390.
6. W. Mulbry, S. Kondrad, C. Pizarro and E. Kebede, (2008). Westhead Treatment of dairy manure effluent using freshwater algae: algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. *Bioresour Technol*., 99: 8137-8142
7. E.J. Olguin, (2003). Phycoremediation: key issues for cost-effective nutrient removal processes**.** *Biotechnol. Adv*., 22 :81-91
8. Lee, K. and Lee, C.-G., (2001). Effect of light/dark cycles on wastewater treatment by microalgae. *Biotechnol. Bioprocess Eng*., 6: 194-199.

9. Lima, S. A., de J. Raposo, M. F., Castro, P. M. L. and Morais, R.M., (2004). Biodegradation of pchlorophenol by a microalgae consortium. *Water Res*., 38: 97-102.
10. Mulbry, W. W. and Wilkie, A. C., (2001). Growth of benthic freshwater algae on dairy manures. *J. Appl. Phycol*., 13: 301-306.
11. Voltolina, D., Gómez-Villa, H. and Correa, G., (2005). Nitrogen removal and recycling by Scenedesmus obliquus in semi continuous cultures using artificial wastewater and simulated light and temperature cycle. *Bioresour. Technol*., 96: 359-362.
12. V.J., Dominic & Murali, Soumya & M.C., Nisha. (2009). Phycoremediation efficiency of three micro algae Chlorella vulgaris, Synechocystis salina and Geloeocapsa gelatiosa. *SB Academic Review*. 16: 138-146.
13. Rao, Polur & Ranjith Kumar, R & G. Raghavan, B & V. Subramanian, V & Sivasubramanian, V. (2011). Application of phycoremediation technology in the treatment of wastewater from leather-processing chemical manufacturing facility. *Water SA*. 37: 07-14.
14. Sharma, Gulshan & Khan, Shakeel., (2013). Bioremediation of Sewage Wastewater Using Selective Algae for Manure Production. *International Journal of Environmental Engineering and Management*,4(6):573-580.
15. Pathak, Vinayak & Singh, D P & Kothari, Richa & Chopra, A., (2014). Phycoremediation of textile wastewater by unicellular microalga Chlorella pyrenoidosa. *Cellular and molecular biology*. 60 (5): 35-40.
16. Kannan V, Vijayasanthi M and Rajmohan N., (2011). Bioremediation of Tannery Effluents by Filamentous Cyanobacteria *Anabaena flos-aquae* West. *Hydrol Current Res*. 2:122.
17. P.S. Lau, Tam N.F.Y. and Y.S. Wong, (2010). Wastewater Nutrients Removal by Chlorella vulgaris: Optimization Through Acclimation. *Environmental Technol.* 2: 183-189.
18. Luz E.G., Rosa O.C., and Sandra Baena, (1997). Efficiency of Ammonia and Phosphorous Removal from a Colombian Agroindustrial Wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. *Bioresource Technol*. 60: 259-262.
19. Okoh, Anthony & Odjadjare, Emmanuel & Igbinosa, Etinosa & N. Osode, Augustina. (2007). Wastewater treatment plants as a source of microbial pathogens in receiving watersheds. *African Journal of Biotechnology*, 6 (25): 2932-2944.
20. Cecal, A., Humelnicu, D., Rudic, V. et al, (2012). *Central European Journal of Chemistry*, 10 (5): 1669-1675.
21. Hwang, Jae-Hoon & Church, Jared & Lee, Seung-Jin & Park, Jungsu & Lee, Woo. (2016). Use of Microalgae for Advanced Wastewater Treatment and Sustainable Bioenergy Generation. Environmental Engineering Science 33(11).
22. Zimmo O.R., Al-Saed R.M., Van der Steen N.P., Gijzen H.J., (2000). Process Performance Assessment of Algae-Based and Duckweed Wastewater Treatment Systems. *Proceedings of the First Latine American Conference on Waste Stabilisation Ponds.*
23. El-Bestawy, Ebtesam & Helmy, Shacker & Hussien, Hany & Fahmy, Mohame & Amer, Ranya. (2013). Bioremediation of heavy metal-contaminated effluent using optimized activated sludge bacteria. *Appl Water Sci*. 3: 181-192
24. Horan N.J., (1990). Biological Wastewater Treatment Systems: Theory and Operations, *Chichester: Wiley.*
25. Cabral JPS., (2010). Water Microbiology. Bacterial pathogens and water. *International Journal Research and Public Health*, 7: 3657-3703.
26. Bitton G., (2005). Microbial indicators of fecal contamination: application to microbial source tracking. Department of Environmental Engineering Sciences, University of

Florida. Gainesville. Report submitted to the Florida Stormwater Association 719 East Park Avenue, Tallahassee, 32301.
27. Matthew N. Campbell, (2008). Biodiesel: Algae as a Renewable Source for Liquid Fuel. *Guelph Engineering Journal* 1: 2-7.
28. Singh, Nirbhay & Dhar, Dolly. (2010). Microalgal Remediation of Sewage Effluent. *Proceedings Indian National Science Academy*. 76: 209-221.
29. Cox, S., Abu-Ghannam, N. and Gupta, S.,(2010). An Assessment of the Antioxidant and Antimicrobial Activity of Six Species of Edible Irish Seaweeds. *International Food Research Journal*, 17: 205-220.
30. Edvardsen, Bente & Skulberg, Randi & Skulberg, Olav. (2004). NIVA Culture Collection of Algae - Microalgae for science and technology. *Nova Hedwigia*, 79: 99-114.
31. Pratt R., T.C. Daniels, J.J. Eiler, J.B. Gunnison and W.D. Kumler et al., (1944). Chlrorellin, an antibacterial substance from Chlorella. *Science*, 99: 351-352.
32. Santoyo S, Rodríguez-Meizoso I, Cifuentes A, Jaime L, García-Blairsy Reina G, Señorans FJ, Ibáñez E., (2009), Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from Haematococcus pluvialis microalgae. *LWT–Food Sci. Technol*, 42: 1213-1218.
33. Younes Ghasemi, Ameneh Moradian , Abdolali Mohagheghzadeh , Shadman Shokravi and Mohammad Hossein Morowvat, (2007). Antifungal and Antibacterial Activity of the Microalgae Collected from Paddy Fields of Iran: Characterization of Antimicrobial Activity of *Chroococcus dispersus*. *Journal of Biological Sciences, 7: 904-910*.
34. Neelam Arun, Shalini Gupta and D.P. Singh, (2012). Antimicrobial and Antioxidant Property of commonly found microalgae *Spirulina platensis*, *Nostoc muscorum*, and *Chlorella pyrenoidosa* against some pathogenic bacteria and fiungi. *International Journal of Pharmaceutical Sciences and Research*, 43: 4866-4875.
35. Vishnu N, and Sumathi R, (2014). Isolation of fresh water microalgae Chlorella sp. and its antimicrobial activity on selected pathogens. *Int J Adv Res BiolSci*; 1: 36-43.
36. Stolz, P. and Obermayer, B. 2005 Manufacturing microalgae for skin care. *Cosmetics Toiletries*, 120: 99– 106.
37. W.J. Oswald (1988). Large scale culture systems (engineering aspects), *Micro-algal Biotechnology, Cambridge Univ. Press*, 357-394.
38. Bosma, Rouke & A. van Spronsen, Wim & Tramper, J & Wijffels, René, (2003). Ultrasound, a new separation technique to harvest microalgae. *Journal of Applied Phycology*, 15(2): 143-153.
39. De la Noue, J. and De Pauw, N., (1988). The Potential of Microalgal Biotechnology. A Review of Production and Uses of Microalgae. *Biotechnology Advances*, 6: 725-770.
40. J. Tampion and M.D. Tampion, (1989). Immobilized Cells: Principles and Applications. *Cambridge Studies in Iniotechnology*, 29 (10): 719.
41. 'Organic Pollutants in Water: Sampling, Analysis, and Toxicity Testing' Issue 214, Division of Environmental Chemistry, American Chemical Society.
42. Hallier, U.W. and R.B. Park., (1969). Photosynthetic light reactions in chemically fixed spinach thylakoids. *Plant Physiol*. 44: 544-546.
43. Travieso, L & Cañizares-Villanueva, Rosa & Borja, R & Benítez, F & Domínguez-Bocanegra, Alma & Dupeyrón R & Valiente V., (1999). Heavy Metal Removal by Microalgae. *Bulletin of contamination and toxicology*, 62: 144-151.

44. Garbisu C, Gil JM, Bazin MJ, Hall DO, and Serra JL, (1991). Removal of nitrate from water by foam-immobilized *Phormidium laminosum* in batch and continuous-flow photobioreactor. *Appl Microbiol Biotechnol* 39: 782-787.
45. Tam NFY, Lau PS, and Wong YS, (1994). Wastewater inorganic N removal and P removal by immobilized *Chlorella vulgaris*. *Water Sci Technol*, 30: 369-374.
46. Gonzalez LE, Canizares RO, and Baena S. (1997). Efficiency of ammonia and phosphorous removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus. Bioresour Technol 60: 259-262*.
47. Mallick N., (2002). Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A Review. *Biometals* 15: 377-390.
48. Murugesan K., (2003). Bioremediation of paper and pulp mill effluents. *Indian J Exp. Bio.* 441:1239-1248.
49. Shashirekha. V., Sridharan M.R., Swamy. M., (2008), Biosorption of trivalent chromium by free and immobilized blue green algae: kinetics and equilibrium studies, *J Environ Sci Health A Tox Hazard Subst Environ Eng*, 43: 390-401.
50. Dubey, S. K.,Dubey, J. Mehra, S. Tiwari, P. Bishwas, A. J. (2011). Potential use of cyanobacterial species in bioremediation of industrial effluents. *African Journal of Biotechnology* 10(7): 1125-1132.
51. Kshirsagar, Ayodhya. (2013). Bioremediation of wastewater by using microalgae: an experimental study. *Int. J. LifeSc. Bt & Pharm. Res*. 02: 339-346.
52. El-Sheekh, Mostafa & Metwally, Metwally & Allam, Nanis & Hemdan, Hany. (2017). Effect of Algal Cell Immobilization Technique on Sequencing Batch Reactors for Sewage Wastewater Treatment. International Journal of Environmental Research. 11(2): 603-611.
53. Bhardwaj, N., (2013). Integrated system of phytodepuration for agro-industrial waste water—An ecofriendly technology. *Recent Res. Sci. Technol*. 2013, 5: 100–102.
54. Bhardwaj RM. 2005. Status of Wastewater Generation and Treatment in India, IWG-Env Joint Work Session on Water Statistics, Vienna, 20-22 June 2005.
55. CPCB. 2009. Status of water supply, wastewater generation and treatment in Class I cities and Class II towns of India. Series: CUPS/70/2009-10. Central Pollution Control Board, India.
56. T.V. Ramachandra, Bharath H. Aithal, Alakananda B., and Supriya G., (2015). Environmental Auditing of Bangalore Wetlands. *ENVIS Technical Report: 72.*
57. Lyon, Stephen & Ahmadzadeh, Hossein & Murry-Ewers, Marcia. (2015). Algae-Based Wastewater Treatment for Biofuel Production: Processes, Species, and Extraction Methods. *Chapter 14: Harvesting and downstream processing and their economics,* 2: 95-115.
58. Li, Yu-Ru & Tsai, Wen-Tien & Hsu, Yi-Chyun & Xie, Meng-Zhi & Chen, Jen-Jeng. (2014). Comparison of Autotrophic and Mixotrophic Cultivation of Green Microalgal for Biodiesel Production. *Energy Procedia*. 52: 371-376.
59. "The Chemical Analysis of Water: General Principles and Techniques", (1986), D.T.E. Hunt, Anthony Leslie Wilson, and A.L. Wilson.
60. Strickland, J.D.H., and Parsons, T.R. (1968). Determination of reactive phosphorus. In: A Practical Handbook of Seawater Analysis. *Fisheries Research Board of Canada, Bulletin* 167: 49–56.
61. "Chemical Methods for Environmental Analysis – Water and Sediment", (1996) by R. Ramesh.
62. McKinney G., (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140:315-322.

63. Khalid, Anwar & Urrehman, (2011). Antimicrobial activity analysis of extracts of Acacia modesta, Artimisia absinthium, Nigella sativa and Saussurea lappa against Gram positive and Gram negative microorganisms. *African Journal of Biotechnology*, 10: 4574-4580.
64. Becker EW., (1994). Microalgae, Biotechnology and Microbiology. *Cambridge University Press*
65. Saikuman Chalivendra, (2014). Bioremediation of wastewater using microalgae. University of Dayton.
66. El-Sheekh, Mostafa & Metwally, Metwally & Allam, Nanis & Hemdan, Hany. (2017). Effect of Algal Cell Immobilization Technique on Sequencing Batch Reactors for Sewage Wastewater Treatment. *International Journal of Environmental Research*. 11(2): 603-311.