Microalgal diversity and its role in wastewater treatment

A thesis submitted
in fulfillment of the requirement for
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In

Biotechnology and Environmental Sciences

Rajiv Kumar

(Regd No. 9051051)

DEPARTMENT OF BIOTECHNOLOGY AND ENVIRONMENTAL SCIENCES

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Content

Certificate

This is to certify that the thesis entitled "Microalgal diversity and its role in wastewater treatment" submitted by Mr. Rajiv Kumar in fulfillment of the requirements for the award of Degree of Doctorate of Philosophy in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of student's own independent and original work carried out by him under my supervision and guidance. The material embedded in this thesis has not been submitted in part or full to any other University for the award of any degree.

Dr. Dinesh Goyal
(Supervisor)
Professor and Executive Director, STEP
Department of Biotechnology and Environmental Sciences
Thapar University
Patiala -147004, Punjab (INDIA)

Declaration

I hereby declare that the work which is being presented in this thesis "Microalgal diversity and its role in wastewater treatment" submitted by undersigned for the award of the degree of Doctor of Philosophy in Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Date: 05/07/2012
Place: Patiala

(Rajiv Kumar)

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Rajiv Kumar

ABSTRACT

Present work highlights the role and application of microalgal diversity in wastewater stabilization ponds with its potential for treating wastewater in terms of removal of organic and inorganic pollutants and heavy metal remediation. *Chlorella* sp. was present as one of the most dominating species in wastewater stabilization pond during whole 10 months on-site study at village Sanghol, Distt. Fatehgarh Sahib, Punjab, India. The order of microalgal dominance was *Chlamydomonas* > *Lyngbya* > *Diatoms*, whereas *Chlorococcum* sp. and *Closteriopsis* sp. were also seen in the month of August and September besides cyanobacteria like *Gloeocapsa* and *Myxosarcina*. A marked reduction of 15 to 83% in BOD_5 and 52 to 93% in COD from inlet wastewater after treatment represented effective treatment potential of wastewater stabilization ponds during complete sampling period. The stabilization ponds also demonstrated their metal remediation potential by reducing 72% and 73% removal of Zn^{2+} and Pb^{2+} especially in the months of November and March respectively. Metal removal studies carried out with pure culture of *Chlorella* sp. (R1) and consortium (CP1) developed from pond wastewater revealed their efficient metal removal potential where *Chlorella* sp. (R1) demonstrated maximum removal potential for Zn^{2+}>Pb^{2+}>Cr (total) whereas, consortium (CP1) removed maximum Zn^{2+} from medium followed by Cr (total) and Pb^{2+}. *Chlorella* sp. (R1) which was found as most dominating in wastewater showed more resistance to metal contamination in comparison to earlier reported isolates with EC_{50} of 4.34 and 10.25 mg L^{-1} for total Cr and Zn^{2+} respectively, whereas Pb^{2+} was non toxic upto 20 mg L^{-1}. Metal uptake capacity (q_{max}) of 33.31, 63.92 mg g^{-1} for Pb^{2+} and Zn^{2+} respectively by algal consortium (CP1) and 34.36, 41.75 and 60.7 mg g^{-1} for Pb^{2+}, Zn^{2+} and total Cr by *Chlorella* sp. (R1) respectively demonstrated good metal uptake potential. *Chlorella* sp. (R1) was analysed for COD and BOD_5 reduction under indoor conditions and it's effective role in removal of Cr from electroplating industrial wastewater using immobilized alga in packed bed column showed its strong potential for development of commercial bioresin.

INTRODUCTION

Uncontrolled discharge of untreated domestic and industrial wastewater contaminates both surface and underground water with high BOD and COD loading whereas, metal processing and electroplating industries add various metals Cd, Cr, Co, Cu, Pb, Mn, Zn, Hg and V in it. With the limited availability and increasing pollution of the ground water, it has become imperative to make the water reusable by removing the pollutants and therefore wastewater treatment has become both an ecological as well as economical necessity.

Presently the application of conventional wastewater treatment systems in countries with low GNP **(**gross national product) is limited because of high cost and technological complexity. Worldwide, there is a continuous interest in algal-based waste stabilization pond systems that are inexpensive and are known for their ability to achieve good removal of pathogens and organic pollutants (Zimmo *et al*., 2002). Cyanobacteria and microalgae play an important role in the system since they supply molecular oxygen to heterotrophic partners and thus support the initial steps of degradation (Cerniglia, 1992). Nutrient removal with aid of algae compares favorably with other conventional technologies (De la Noue *et al*., 1992; Raghukumar *et al*., 2001; Pinto *et al*., 2002; Muthukumaran *et al*., 2005) moreover, some cyanobacteria and algae might remove xenobiotics from the environment by sorption, transformation and degradation (Olguín, 2003). However, high algal concentration of about 100 mg TSS L^{-1} may be occasionally reached in the effluent, causing severe clogging problems in drip irrigation system (Pearson *et al*., 1995). Treatment of both domestic and agricultural wastewater by algae has been investigated thoroughly by Oswald in 1988, and several attempts have been made to explore the efficiency of microalgae for metal removal (Wilde and Benemann, 1993; Zayed and Terry, 2003; Chu and Hashim, 2004). It is obvious that a system consisting of several micro-organisms is preferable in bioremediation processes since it is nearly impossible to find a microorganism species that can degrade a mixture of different pollutants completely by itself (Alexander, 1994). The performance of an oxidation pond depends on the biotic community present and pollutants (Wong *et al*., 1995) from which phytoplankton of wastewater lagoons are quite significant **(**Oswald, 2003) but there is little data available on the structure and seasonal variation of micro-algal community of wastewater treatment ponds.

Algae are also well known for their capacity to accumulate metals from wastewater since many heavy metals e.g Cu, Fe, Mn, Zn, Co and Mo are required as essential micronutrients (Harish *et al*., 2008). Algae present in facultative ponds act as a dominant contributor in removal of heavy metals by surface adsorption and bioaccumulation. In this context, accumulation of metals by microorganisms, including algae, has been known for a few decades has received increased attention only in recent years because of its potential for application in environmental protection or recovery of precious or strategic metals (Malik, 2004). Metal accumulation capacity of algal biomass is either comparable or sometimes higher than chemical sorbents. However, the fragile nature of live algae is not suitable for robust wastewater treatment operations with other essential requirement of an industrial sorption system is that the sorbent can be utilized as a fixed or expanded bed which led to the interest of using entrapped biomass in immobilized preparations. In this context, immobilization of algal cell for wastewater treatment has been proposed for circumventing the harvest problems, as well as retaining the high-value algal biomass for further processing (Fogarty *et al*., 1999). Therefore, algal biomass may serve as an economically feasible and efficient alternative to the existing physicochemical methods of metal removal and recovery from wastewater. While the accumulation of single species of heavy metal ions by algal biomass has been extensively studied, very little attention has been given to the study of multi-metal system especially with live algae whereas many industrial wastewaters contain high level of more than one metal.

In the present investigation a linkage between systems ecology of microalgae present in wastewater stabilization ponds and its potential of treating wastewater in terms removal of organic and inorganic pollutants and heavy metal remediation has been worked out. A comprehensive study was undertaken to explore the role and potential of microalgae found in wastewater ponds for wastewater treatment and heavy metal Pb^{2+}, Zn^{2+} and total Cr removal from wastewater, with following objectives.

OBJECTIVES

1. Monitoring of microalgal diversity of wastewater
2. Removal of metal (Zn^{2+}, Pb^{2+} and total Cr) by selected microalgae
3. Development of strategy for wastewater treatment using microalgae

Approach adopted to meet above objectives

Ten months on-site study was carried out (August 2004 to May 2005) to study the wastewater treatment potential of a waste stabilization pond situated at village Sanghol, Distt. Fatehgarh Sahib, Punjab, India. Periodic wastewater samples were collected after an interval of one month from inlet and outlet pond streams and analyzed for in water quality parameters. Wastewater samples were studied microscopically for the presence of different microalgal types and their relative abundance during complete observation period. Unialgal cultures consisting of pure single culture and mixed algal culture consisting two or more algal species were developed from wastewater using stab and serial plating techniques. Algal consortium and pure culture of *Chlorella* sp. (R1) were studied for metal removal (Pb^{2+}, Zn^{2+} and total Cr) and uptake potential both from single and bi-metallic aqueous solution [Pb^{2+}-Zn^{2+}, Pb^{2+}-Cr (total) and Zn^{2+}-Cr (total)]. An indoor study of wastewater treatment was also carried out under tub conditions filled with diluted wastewater to monitor the role of inoculated microalgae over a period of three months. In another strategy to treat metal contaminated wastewater, *Chlorella* and algal consortium were mass cultivated and immobilized on silica gel and characterized for their metal sorption potential from synthetic metal solutions under packed bed conditions.

1. Monitoring of microalgal diversity of wastewater

Characterization of wastewater

Ten month onsite case study from August 2004 to May 2005 was carried out to monitor the potential of algal based wastewater stabilization ponds in treatment of domestic wastewater generated from the village of Sanghol Distt. Fatehgarh sahib, Punjab, India. The domestic wastewater was applied directly to the pond 1 after passing through an equalization tank where the algae and duckweeds in pond 1 (Facultative pond) utilise inorganic nutrient for their growth by lowering COD and nascent oxygen produced during photosynthesis augments complete oxidation of organic compounds resulting in lowering of BOD_5. Pond inlet and outlet wastewater samples were periodically characterized at monthly interval for physicochemical parameters (temperature, pH, conductivity, salinity, dissolved oxygen, chemical oxygen demand and biochemical oxygen demand) alongwith enumeration of culturable bacteria and metals (Pb^{2+}, Zn^{2+} and total Cr) to monitor wastewater treatment potential of pond system. The temperature varied from minimum 12.7 and 12.0 °C for inlet and outlet wastewater in the month of January whereas

maximum temperature of 32 to 33.6 °C was observed in month of August. The pH of the system varied from 7.83 to 10.72 where as conductivity range from 1.75 to 4.67 mS. Salinity of wastewater was in the range of 1 to 2.3 ‰ whereas dissolved oxygen varied widely from 14 mg L^{-1} indicating complete saturation of water to 0.55 mg L^{-1} showing limitation of oxygen in wastewater. The COD and BOD_5 showed marked variation during complete study period, where removal of both COD and BOD_5 in outlet water samples in comparison to inlet samples demonstrated pond treatment potential.

Microalgal diversity

The diversity of microalgae was studied in wastewater samples microscopically over complete observation period, where algal species were identified based on identification keys following monographs on algae (Smith, 1950; Prescott, 1951; Wehr and Robert, 2003). Wastewater was characterized for the relative dominance of algal species and it was observed that *Chlorella* sp. was present during the whole sampling period as one of the most dominating species followed by *Chlamydomonas*>*Lyngbya*>*Diatoms*, whereas *Chlorococcum* sp. and *Closteriopsis* sp. were also seen in the month of August and September. Cyanobacteria like *Gloeocapsa* and *Myxosarcina* were also reported in wastewater. Two unialgal cultures (R1, R2) and five consortia of mixed algal culture (R3, R5, R6, R9 and CP1) were developed by isolating algae from wastewater samples collected from algal stabilization pond using classical stab technique and serial plating on solid BG-11 media plates. Algal cultures R1 and R2 were identified as pure cultures of *Chlorella* sp. and *Chlorococcum* sp. respectively where as, algal culture R3 was found to be a mixture of *Chlorella* and *Chlorococcum*. The other algal cultures R5 was found to contain *Nostoc* sp. in association with *Chlorella* sp. whereas R6 contained mixture of three cyanobacteria (*Myxosarcina* sp., *Gloeocapsa* sp. *and Chlamydomonas* sp.) and R9 contained only *Myxosarcina* sp., *Gloeocapsa* sp. Consortium (CP1) consisting of mix algal composition dominated by *Chlorella* > *Chlamydomonas* > *Lyngbya* sp. was developed in liquid BG-11 medium by enrichment technique.

Onsite treatment potential

There was marked difference between the water characteristics of pond inlet and outlet wastewater, where pH of the water was alkaline through out, however it tends to become more alkaline after treatment. The temperature of the water was lowest in the month of January, which was 12 ^{o}C and it was 32 ^{o}C in the month of August and September

whereas conductivity of wastewater was decreased after treatment which ranged from 1.9 to 4 for untreated wastewater and 1.7 to 2 for treated wastewater with similar reduction in salinity. The BOD_5 and COD values were higher in the influent water than the treated wastewater. A marked reduction of 15 to 83% in BOD_5 and 52 to 93% in COD after treatment represented effective wastewater treatment potential of facultative pond. The viable bacterial cell count in terms of cfu ml^{-1} was drastically reduced in wastewater after treatment however it was higher during the month of December and January.

2. Removal of metal (Zn^{2+}, Pb^{2+} and total Cr) by selected microalgae *Chlorella* sp. (R1) and algal consortium (CP1)

Two unialgal cultures (R1, R2) and five mixed algal cultures (R3, R5, R6, R9 and CP1) were initially screened for their ability to remove metals (Pb^{2+}, Zn^{2+} and total Cr) during growth, for which cultures were grown in 100 ml BG-11 medium containing single metals at fixed initial concentration of 5 mg L^{-1}. Algal cultures R1, R2 and R5 more than Pb^{2+} removal from culture medium after 12 days of incubation. All algal cultures except R9 were found as good candidates for Zn^{2+} removal. Consortium CP1 was found to remove 68% Zn^{2+} followed by *Chlorella* sp. (R1) with 67% removal. All the seven algal cultures had least ability to remove chromium which was only 10-40%. *Chlorella* sp. (R1) which was observed as the most dominating microalgae present in pond system and consortium (CP1) which represented the microalgal diversity developed denovo from pond water were selected to study the effect of varying concentration of Pb^{2+}, Zn^{2+} and total Cr on the growth and metal removal potential.

Effect of metal on growth

The pure culture of *Chlorella* sp. (R1) showed 3 days of lag phase followed by 10 days of exponential growth phase in BG-11 medium under controlled laboratory conditions. The effect of varying concentration of metals (Pb^{2+}, Zn^{2+} and total Cr) was studied following the unicellular algal cell count using haemocytometer and chlorophyll content as indicator of growth. Pb^{2+} demonstrated positive effect of increasing Pb^{2+} concentration on *Chlorella* cell number which increased from 118 x 10^5 to 278 x 10^5 cells ml^{-1} by increasing external metal concentration from 1 to 20 mg L^{-1}. Cell number was slightly increased from 432 x 10^5 to 538 x 10^5 cells ml^{-1} by increasing Zn^{2+} concentration from 1 to 5 mg L^{-1}, whereas EC_{50} for Zn^{2+} was calculated to be 10.25 mg L^{-1} by probit analysis (Finney, 1952). Effect of varying total Cr concentration on cell count after 12 days of incubation revealed that 1

mg L^{-1} of total Cr stimulates the growth of *Chlorella* sp. (R1) while there is a drastic reduction in cell count at 5 mg L^{-1} with EC_{50} value of 4.34 mg L^{-1}. The cell count of 5.3 x 10^5 to 2.5 x 10^5 was maintained by increasing total Cr concentration from 5-20 mg L^{-1}, showing the survival of resistant cells at higher metal concentration.

The chlorophyll content of consortium (CP1) was analysed as measure of growth in medium containing heavy metals (Pb^{2+}, Zn^{2+} and total Cr). Lead exhibited positive effect on growth of algal consortium (CP1) at 10 mg L^{-1} of Pb^{2+} over its non-metallic control chlorophyll content increased from 3 x 10^{-4} to 6.9 x 10^{-4} mg ml^{-1}, however the growth was inhibited at 20 to 50 mg L^{-1} of lead. Zn^{2+} was more toxic to consortium (CP1) than Pb^{2+}, as there was reduction in chlorophyll content by 90.5% at 10 mg L^{-1} of Zn^{2+} over non-metallic control. This reduction in chlorophyll content was continued by further increase in external Zn^{2+} concentration. Chromium was even more toxic metal to algal consortium (CP1) by inhibiting growth even at 1 mg L^{-1} of total Cr where 75% reduction in chlorophyll content was observed by increasing total Cr from 1 to 5 mg L^{-1}.

The effects of three heavy metals (Pb^{2+}, Zn^{2+} and total Cr) were also studied on the growth by *Chlorella* sp. (R1) under bimetallic conditions of Pb^{2+}-Zn^{2+}, Pb^{2+}-Cr (total) and Zn^{2+}-Cr (total). The order of metal toxicity on *Chlorella* sp. (R1) cell number was Cr (total) > Zn^{2+} > Pb^{2+}.

Metal removal

Both algal consortium (CP1) and *Chlorella* sp. (R1) demonstrated their prospective ability in removing heavy metals (Pb^{2+}, Zn^{2+} and total Cr) from wastewater as observed from metal removal experiments conducted under controlled laboratory conditions. Consortium (CP1) revealed its maximum ability for removing Zn^{2+} from BG-11 medium during its 15 days of growth, which varied from 68-73% at an optimum external Zn^{2+} concentration of 1-20 mg L^{-1}. The metal removal potential by consortium (CP1) was Zn^{2+} > Cr (total) > Pb^{2+}, with 25% total Cr and 17% Pb^{2+} were removed from medium at an optimum concentrations of 5 and 10 mg L^{-1} respectively. The removal efficiencies for all the three metals were reported to decline by further increasing external metal concentration.

In a similar study *Chlorella* sp. (R1) also demonstrated its maximum removal potential for Zn^{2+} and Pb^{2+} by removing 60-70% Zn^{2+} from medium containing 5-20 mg L^{-1} of external metal concentration, whereas 66% Pb^{2+} was removed at an optimum concentration of 1 mg L^{-1}. 40 to 50 % Pb^{2+} removal efficiency was maintained by further increasing medium Pb^{2+} concentration by 5-20 mg L^{-1}. *Chlorella* sp. (R1) demonstrated

least removal potential for total Cr by removing only 10 % total Cr from medium containing 1-5 mg L^{-1} of total Cr concentration whereas in an exceptional observation this removal efficiency suddenly increased from 10 to 67% in medium containing 10 mg L^{-1} of total Cr which was maintained with slight reduction at 44% at 20mg L^{-1}. Metal removal experiments conducted on bimetallic solution with *Chlorella* sp. (R1) showed effect of one metal on removal of other metal, where Pb^{2+} removal efficiency was increased in the presence of Zn^{2+} which increased by 56 to 98% on increasing Zn^{2+} concentration, whereas Zn^{2+} removal declined by increasing Pb^{2+} concentration in the medium. Zn^{2+} and Pb^{2+} removal was also negatively effected by presence of total Cr where 15 to 20% decline in maximum Zn^{2+} removal efficiency was observed by increasing total Cr concentration from 1 to 20 mg L^{-1} with similar decline in Pb^{2+} removal also. Same poor total Cr removal capacity of *Chlorella* sp. (R1) varied from 5 to 20% was also monitored from bimetallic mixture containing Cr (total) with Pb^{2+} or Zn^{2+} also, which further declined by increasing Pb^{2+} and Zn^{2+} concentrations.

Metal uptake potential

Both algal consortium (CP1) and pure culture of *Chlorella* sp. (R1) had significant metal uptake potential. Metal uptake capacity (q_{max}) of 33.31, 63.92 mg g^{-1} was calculated for Pb^{2+} and Zn^{2+} respectively by mass balance for live algal consortium (CP1) whereas q_{max} for *Chlorella* sp. (R1) was 34.36, 41.75 and 60.7 mg g^{-1} for Pb^{2+}, Zn^{2+} and total Cr respectively. Metal uptake capacity was found to increase per gram of *Chlorella* sp. (R1) biomass from medium containing bimetallic mixtures.

3. Development of strategy for wastewater treatment using microalgae

Indoor studies on wastewater treatment using microalgae

To study the role of microalgae in wastewater treatment a time course study was carried on laboratory scale to compare the changes in wastewater characteristics by inoculated microalgae. Experiment was set in four round plastic tubs of 10 L capacity, each filled with 8 L wastewater diluted by liquid BG-11 medium mixed in 1:3. Four tubs were divided into two sets of 2 each, from which tub 1 of each set was kept as control, whereas tub 2 was inoculated with 50 ml log phase culture of *Chlorella* sp. (R1). One set of two tubs was kept in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent lamps where as other set was kept under natural outdoor conditions of light and atmosphere. Representative samples from each tub

were drawn at an interval of ten days and characterized for temperature, pH, electrical conductivity, salinity, chemical oxygen demand and five day biochemical oxygen demand. Loss of water by evaporation was maintained by addition of sterile distilled water.

The study revealed effective role of microalgae developed denovo in wastewater treatment with major contribution of *Chlorella* sp. (R1) as most dominating algae. The comparative advantage of changing environmental conditions with mid day water temperature varying from 29.7 to 10.5 °C for outdoor tubs over controlled laboratory conditions with constant temperature was clearly observed in terms of continuous decline in conductivity and salinity with passage of time for outdoor tubs that indicated removal of soluble salts. Role of microalgae in reduction of wastewater COD was observed by comparing COD reduction data of indoor tubs after 80 days, where tub 2 inoculated with *Chlorella* sp. (R1) removed 223.7 mg L^{-1} of COD in comparison to tub 1 (control) with respective COD removal values of 158.7 mg L^{-1}. Outdoor colder climate conditions showed negative effect on COD removal where only small reduction of 185.6 and 153.6 mg L^{-1} was reported from tubs 1 and 2 respectively, showing the role of microalgae developed denovo and *Chlorell*a sp. (R1) as dominant contributor in COD removal. Similarly 63.7 and 73.9 mg L^{-1} reduction in BOD_5 was monitored for tubs 1 and 2 respectively placed indoor, where as these reductions by 63.7 and 73.9 mg L^{-1} for respective two outdoor tubs showed role of *Chlorella* sp. (R1) in wastewater treatment

Metal removal in packed bed columns

Chlorella sp. (R1) was mass cultivated, dried and immobilized on silica gel and used in packed bed columns to strip metals Pb^{2+}, Zn^{2+} and total Cr from synthetic metal solutions. The comparative metal removal ability for 2 g biomass packed in plastic column and gravity fed with metal solutions achieving 4.3 ml min^{-1} flow rate demonstrated maximum removal potential for total Cr by removing 100% total Cr from inlet metal synthetic solution (Cr 92.6 mg L^{-1}, pH 5.7) where as these reductions were 96.4% and 86% in first 5 ml sample collected from outlet stream fed by Pb^{2+} (Pb^{2+} 156.6, pH 4.3) and Zn^{2+} (Zn^{2+} 82.6, pH 5.5) synthetic solutions respectively. The metal uptake capacity of 1.07, 5.24 and 1.27 mg g^{-1} of immobilized biomass for total Cr, Pb^{2+} and Zn^{2+} as calculated for solution mass balance indicate the metal binding sites available whereas maximum recovery 65%, 87% and 58% with 0.12N HCl for corresponding total Cr, Pb^{2+} and Zn^{2+} revealed maximum strength of Zn^{2+}>Cr (total)>Pb^{2+} ion binding with algal biomass. Breakthrough curves obtained for percent metal removal against volume of solution passed, where the

distance of saturation point from origin determine the usability of column biomass showed maximum usability of *Chlorella* sp. (R1) biomass for Pb^{2+}>Cr(total)>Zn^{2+}. The role of increase in flow rate as achieved by reducing biomass per column resulted into drastic reductions in column performance.

Chlorella sp. (R1) biomass under immobilized condition was also found capable of removing chromium from wastewater streams of chrome electroplating industry in packed bed columns where two columns packed with 2g immobilized biomass exhibited 80.07 % total Cr removal respectively from chrome electroplating industrial wastewater (Cr 341 mg L^{-1}, pH 1.44) with 80% recovery of total Cr by dilute HCl. Biomass also showed good total Cr uptake of 22.97 mg per gram of *Chlorella* sp. (R1) biomass where as silica acted as an effective supporting material for immobilization.

In a similar study 1.5g algal consortium (CP1) immobilized in silica gel and packed in column demonstrated maximum 92.5% Pb^{2+} removal from Pb^{2+} synthetic solution (Pb^{2+} 33.90 mg L^{-1}, pH 4.3) with 80% lead recovery with 0.12N HCl.

Salient findings

1. Present investigation revealed linkage between systems ecology of microalgae present in wastewater stabilization ponds and its potential of treating wastewater in terms of removal of organic and inorganic pollutants and heavy metal remediation.
2. *Chlorella sp.* (R1) was present during the whole sampling period as one of the most dominating species in pond water followed by *Chlamydomonas, Lyngbya, Diatoms,* whereas *Chlorococcum sp*. and *Closteriopsis sp.* were also seen in the month of August and September besides cyanobacteria like *Gloeocapsa* and *Myxosarcina*.
3. Seasonal variations with respect to changes in pH, temperature and light intensity were reflected in abundance and activity of specific group of microalgae in multi-species microbial communities.
4. The pond system showed effective role in wastewater treatment by removing 15 to 83% BOD_5 and 52 to 93% COD, whereas cold climate was found lesser favorable for phycoremediation due to the poor light availability and low temperature.
5. The pond system reduced conductivity and salinity by 47% and 48% respectively of inlet wastewater leading to salinity of 2.12 mS cm^{-1} in outlet water which is good for irrigation purposes.
6. *Chlorella* (R1) had maximum removal potential for Zn^{2+}>Pb^{2+}>Cr (total) whereas Consortium (CP1) had Zn^{2+} > Cr (total) > Pb^{2+} from medium.
7. Removal of metal by *Chlorella* sp. (R1) from bimetallic solutions {Zn^{2+}-Pb^{2+}, Pb^{2+}-Cr (total), Zn^{2+}-Clr(total)} showed interaction among metal ions which resulted in either decrease in removal efficiency or enhancement of removal of one metal in presence of other.
8. Cr was more toxic to *Chlorella* sp. (R1) with EC_{50} of 4.34 mg L^{-1} followed by Zn^{2+} with LC_{50} of 10.25 mg L^{-1} as calculated by probit analysis, whereas Pb^{2+} was nontoxic upto 20 mg L^{-1}.
9. *Chlorella* sp. (R1) was found to have metal uptake capacity (q_{max}) of 34.36, 41.75 and 60.7 mg g^{-1} for Pb^{2+}, Zn^{2+} and total Cr respectively.
10. Efficient contribution of microalgae was observed from microcosm experiments in reduction of COD and BOD_5 with distinct role of *Chlorella* sp. (R1).

11. *Chlorella* (R1) biomass immobilized in silica as supporting material was effective in striping Pb^{2+} from synthetic solution and total Cr from electroplating industrial wastewater.
12. Silica gel was an effective support material for immobilization of algal biomass which provided mechanical strength and chemical stability and this approach can be applied for large scale treatment of industrial wastewater.

List of Abbreviations

rpm	Revolution per minute
g	Gram
mg	Milligram
µg	Microgram
L	Litre
µS cm^{-1}	Microsiemens per cm
ml	Millilitre
µl	Microlitre
mg ml^{-1}	Milligrams per millilitre
µg ml^{-1}	Micrograms per millilitre
%	Percentage
mg L^{-1} (ppm)	Milligrams per litre (parts per million)
µg L^{-1} (ppb)	Micrograms per litre (parts per billion)
mg g^{-1}	Milligrams per gram
µg g^{-1}	Microgram per gram
cm min^{-1}	Centimeter per minute
g L^{-1}	Grams per litre
(x10^3 cfu ml^{-1} wastewater)	x 10^3 colony forming unit per millilitre of wastewater
WSP	Wastewater stabilization pond
HRAP	High rate algal pond
AIWPS	Advanced integrated wastewater pond system
EC	Electrical conductivity
TS	Total solids
TDS	Total dissolved solids
TSS	Total suspended solids
COD	Chemical oxygen demand
BOD	Biochemical oxygen demand
BOD_5	Five day Biochemical oxygen demand
DO	Dissolved oxygen
q_{max}	Maximum metal uptake
R	Bioremoval efficiency
C_i	Initial Concentration of metal in aqueous solution
C_f	Final Concentration of metal in aqueous solution
V	Volume of culture media
S	Sulphur
Pb	Lead
Cr	Chromium
Zn	Zinc
Fe	Iron
Ni	Nickel

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INTRODUCTION

Industrial activities release huge amount of wastewater with high BOD, COD loading whereas, metal processing and electroplating industries add various heavy metals to it. Heavy metals (Cd, Cr, Co, Cu, Pb, Mn, Zn, Hg and V) in the environment have become a serious health problem worldwide due to their incremental accumulation in the food chain through continued persistence in the ecosystem (Dudka and Miller, 1999). With the limited availability and increasing pollution of the ground water, it has become imperative to make the water reusable by removing the pollutants and therefore wastewater treatment has become both an ecological as well as economical necessity. With increasing environmental awareness and the toughening of government policies, it has become necessary to develop new environmental friendly ways to clean up contaminants using low-cost methods and materials (Chandra Sekhar *et al*., 2004).

Presently the application of conventional wastewater treatment systems in countries with low GNP **(**gross national product) is limited because of high cost and technological complexity. Worldwide, there is a continuous interest in algal-based waste stabilization pond systems that are inexpensive and are known for their ability to achieve good removal of pathogens and organic pollutants (Zimmo *et al*., 2002). Treatment of both domestic and agricultural wastewater by algae has been investigated thoroughly (Oswald, 1988) alongwith several attempts have been made to explore the efficiency of microalgae for metal removal (Chu *et al*., 1997; Zayed and Terry, 2003). Cyanobacteria and microalgae play an important role in the system since they supply molecular oxygen to heterotrophic partners and thus support the initial steps of degradation (Cerniglia, 1992). Nutrient removal with aid of algae compares favorably with other conventional technologies (De la Noue *et al*., 1992; Raghukumar *et al*., 2001; Pinto *et al*., 2002; Muthukumaran *et al*., 2005) moreover, some cyanobacteria and algae might remove xenobiotics from the environment by sorption, transformation and degradation (Olguín, 2003). During the last few years, the capability of algae to biotransform and biodegrade phenols (Pinto *et al*., 2002) crude oil (Raghukumar *et al*., 2001), heavy metals like Cr, Pb Zn (Wilde and Benemann, 1993) and other pollutants have been reported with potential in the biotreatment of polluted water. It is obvious that a system consisting of several micro-organisms is preferable in bioremediation processes since it is nearly impossible to find a microorganism species that can degrade a mixture of different pollutants completely by itself (Alexander, 1994). The performance of an oxidation pond depends on the biotic

community present and pollutants (Wong *et al*., 1995) from which phytoplankton of wastewater lagoons are quite significant **(**Oswald, 2003) but there is little data available on the structure and seasonal variation of micro-algal community of wastewater treatment ponds.

Algae are also well known for their capacity to accumulate metals from wastewater since many heavy metals e.g Cu, Fe, Mn, Zn, Co and Mo are required as essential micronutrients (Harish *et al*., 2008). Algae present in facultative ponds act as a dominant contributor in removal of heavy metals by surface adsorption and bioaccumulation. In this context, accumulation of metals by microorganisms, including algae, has been known for a few decades has received increased attention only in recent years because of its potential for application in environmental protection or recovery of precious or strategic metals (Malik, 2004). Conventional precipitation technology and ion exchange resins are still used in many applications for metal removal from wastewater, but are expensive, labor intensive and ineffective at low concentration of metals (Atkinson *et al*., 1998). Recent comparison has also suggested that biosorbent developed from microalgae could be cheaper to implement than other commercially available ion exchange resins (Lloyd, 2002). A few algal strains have been identified that show very strong adsorption affinity for Hg, Au, Cu, Zn, Pb and Cr where metal accumulation capacity of algal biomass is either comparable or sometimes higher than chemical sorbents (Chojnacka *et al*., 2004 ; Ting *et al*., 1995; Mehta *et al*., 2002; Pradhan *et al*., 1998; Sandau *et al*., 1996; Sheng *et al*., 2005). However, the fragile nature of live algae is not suitable for robust wastewater treatment operations with other essential requirement of an industrial sorption system is that the sorbent can be utilized as a fixed or expanded bed which led to the interest of using entrapped biomass in immobilized preparations. Immobilized biomass within or on inert matrix has the inherent advantage that high flow rate can be achieved with minimal clogging of the column, and also that the control size of sorbent particle and high biomass loading of the column reactor are feasible (Yakubu and Dudeney, 1986). Darnall and his co-workers (1986) reported their results using silica to immobilize algal cells for use in "AlgaSORB" columns that are employed in remediation of industrial streams. Therefore, algal biomass may serve as an economically feasible and efficient alternative to the existing physicochemical methods of metal removal and recovery from wastewater.

In the present investigation a linkage between microalgal diversity present in wastewater stabilization ponds and its potential for treating wastewater in terms removal of organic and inorganic pollutants and heavy metal remediation has been worked out. A

comprehensive study was undertaken to explore the role and potential of microalgae present in wastewater ponds for waste water treatment and metal (Pb^{2+}, Zn^{2+} and total Cr) removal from wastewater, with following objectives:

OBJECTIVES

1. Monitoring of microalgal diversity of wastewater
2. Removal of metal (Zn^{2+}, Pb^{2+} and total Cr) by selected microalgae
3. Development of strategy for wastewater treatment using microalgae

REVIEW OF LITERATURE

1. Background

Uncontrolled discharge of untreated domestic and industrial wastewater contaminates both surface and underground water by releasing huge amount of wastewater with high BOD and COD loading whereas, metal processing and electroplating industries add various heavy metals (Cd, Cr, Co, Cu, Pb, Mn, Zn, Hg and V) in it resulting in high levels of water pollution. With the limited availability and increasing pollution of the ground water, it has become imperative to make the water reusable by removing the pollutants and therefore wastewater treatment has become both an ecological as well as economical necessity.

Management of domestic and industrial wastewater has traditionally focused on the reduction of organic and inorganic loading, pathogen elimination and metal removal. The approach has been to address these through technological options; most of them high tech, land requiring and needs to dispose of high quantities of industrial and domestic sewage water and toxic sludge (Mehta and Gaur, 2005). However, emergence of smaller urbanizing areas of predominantly poor and middle class populations of countries with low GNP reduces the economy of scale advantages and has often made such technologies uneconomical and unsustainable (Zimmo *et al*., 2002). Modern researchers are now advocating for natural treatment methods coupled with recovery and reuse of water and nutrients as a decentralized approach of wastewater management where as conventional metal removal techniques are being replaced by biological metal remediation processes. Such natural "green" systems are often intended to describe processes that depend primarily on their natural components to achieve the intended purpose. They might typically include pumps and piping for waste conveyance but would not depend exclusively on external energy sources to maintain the major treatment responses (Reed *et al*., 1995).

Cyanobacteria and microalgae play an important role in natural systems for wastewater remediation since they supply molecular oxygen to heterotrophic partners and thus support the initial steps of degradation (Cerniglia, 1992). Nutrient removal with aid of algae compares very favorable to other conventional technologies (De la Noue *et al*., 1992; Raghukumar *et al*., 2001; Pinto *et al*., 2002; Muthukumaran *et al*., 2005) moreover, some cyanobacteria and algae might remove xenobiotics from the environment by sorption, transformation and degradation (Olguín, 2003). Treatment of both domestic and agricultural wastewater by algae has been investigated thoroughly by Oswald in 1988

(Oswald, 1988), where in several attempts have been made to explore the efficiency of microalgae for metal removal (Wilde and Benemann, 1993; Zayed and Terry, 2003; Chu and Hashim, 2004).

This review compiles the natural "Green" methods available for bioremediation of wastewater and role of microalgae in aquatic systems. The potential of microalgae is also discussed in terms of their metal removal capacity from wastewater.

2. Natural treatment systems

Natural "green" systems are often intended to describe processes that depend primarily on their natural components to achieve the intended purpose. They might typically include pumps and piping for waste conveyance but would not depend exclusively on external energy sources to maintain the major treatment responses (Reed *et al*., 1995). Natural systems follow 'the logic of nature'. In nature the resources are recycled and it may, therefore, be beneficial to replace the 'linear' view on sanitation i.e. collection, transport, centralized treatment and final disposal, with a 'loop approach'. Though sewage may be a 'resource in the wrong place', it has economic value and, therefore, could beneficially be recovered or recycled (King, 2000). In a loop approach, wastewater is seen as a resource.
The general features of natural systems include:

- Natural systems are aimed at recycling of nutrients, water and energy,
- Natural systems use aerobic and/or anaerobic microbiological processes to remove BOD/COD without the need of energy input.
- The oxygen for aerobic microbiological processes in natural system is supplied by photosynthesis (algae, plants) or natural re-aeration.

Natural systems for effective wastewater treatment are available in three major categories: terrestrial, wetland and aquatic concepts (Reed *et al*., 1995; Tchobanoglous, 1997).

2.1 Terrestrial systems

The terrestrial systems are divided into three basic concepts: slow-rate irrigation, rapid infiltration percolation and overland flow. Each method can produce renovated water of different quality, can be adapted to different site conditions, and can satisfy different overall objectives (Qasim, 1994). Irrigation is most widely used form of land treatment system which demands a relatively large area and can be used for fast growing energy crops such as *Salix* sp. (Qasim, 1994). The other two concepts are characterized by a

subsurface vertical flow. In overland flow systems, where water is allowed to percolate through a thin litter layer over a sloping planted surface and biochemical oxidation, sedimentation, filtration and chemical adsorption are the primary mechanisms for removal of contaminants (Wittgren and Hasselgren, 1992). In rapid-infiltration-percolation the wastewater percolates through the soil and treated effluent reaches ground water or underdrain systems which can be recovered by pumping system. Infiltration units are the most commonly recommended and used treatment method for treatment of wastewater from single households also where effluent from septic tanks percolates through a buried bed consisting of a nutrient poor substrate, such as washed macadam leading to adsorption of phosphorus, removal of nitrogen and degradation of organic matter (SNV, 1997; 2002). Construction of an infiltration unit is dependent on the land profile and characteristics of the soil. If the natural soil is unsuitable, then sand filters can be used. Sand filters resemble infiltration units, but usually have two spreading layers and an additional layer of sand, and therefore a defined limited volume can be treated (SNV, 1998).

2.2 Wetland systems

Wetlands are defined as land where the water table is at, or above, the ground surface (usually 0.6 m or more) long enough each year to maintain saturated soil conditions and growth of related vegetation (Reed *et al*., 1995). The vegetation provides surface for attachment of bacterial films, and aids in filtration and adsorption of wastewater constituents. Vegetation also trans-locate oxygen from leaves to the root systems to support a wide range of aerobic and facultative bacteria and controls the growth of algae by restricting the sunlight. Wetland can be of two types: natural wetlands and constructed wetlands which have been used for wastewater treatment, although the use of natural wetlands is generally limited to the polishing or further treatment of secondary or advanced wastewater treated effluent (Healy and Cawley, 2002). Furthermore, the principal objective when discharging to natural wetlands should be enhancement of existing habitat so must meet applicable regulatory requirements for inlet polluted wastewater. Constructed wetlands offer all of the treatment capabilities of the natural wetland but without the constraints associated with the discharging to a natural ecosystem so not restricted for any influent quality (Healy and Cawley, 2002). The type of wetland used is determined by the required treatment, i.e. secondary of tertiary, mass loading, climate, available land and cost (Greenway, 2003).

There are two main categories of constructed wetlands:

2.2.1 Free water surface flow (FWS) wetlands

Free water surface flow wetlands typically consist of shallow vegetated channels or basins with some type of barrier to prevent seepage, soil to support vegetation and water through the system at a relatively shallow depth. Pretreated wastewater is applied continuously to such systems, and the treatment occur as the water flows slowly to the stems and roots of emergent vegetation so they are typically used to provide tertiary treatment after conventional secondary treatment, which removes most of the organic pollutants (Greenway, 2003).

2.2.2 Subsurface flow (SF) wetlands

Subsurface flow wetlands, also called reed beds or root-zone systems, are gravel, sand, or soil filled channels, or basins with horizontal flow of water. The design of these systems assumes that the water level in the bed will remain below the top of the rock or gravel media. The plants serve multiple purposes in wastewater treatment wetlands, and the selection of plant species depends upon the local availability of species, the type of wetland and the chemical composition of the wastewater effluent (Greenway, 2003). SF shows comparative advantage over FWS as if the water surface is maintained below the media surface there is little risk of odors, public exposure, or insect vectors. In addition, it is believed that the media provides larger available surface area for attached growth of organisms. Oxygen leakage from roots creates oxidized conditions which stimulates both aerobic decomposition of organic matter and growth of nitrifying bacteria in the rhizosphere (Brix, 1994; Greenway, 2003).

2.3 Aquatic systems

An aquatic treatment system is defined as the use of aquatic plants or animals as a component in a wastewater treatment system where the major biological components include floating plants, fish and other animals, planktonic organisms and submerged plants (Reed *et al*., 1998). The treatment responses are due either to the direct uptake of material by the plants or animals, and by the presence of this biota altering the physical environment in the system. In addition the vegetation act as host substrate for attached microbial organisms, which provide a very significant degree of treatment (Reed *et al*., 1995). Aquatic systems are further subdivided to distinguish between lagoon and pond

systems depend on microbial life and the lower forms of plants and animals (Reed *et al*., 1995).

2.3.1 Wastewater stabilization ponds

Wastewater stabilization pond (WAP) technology is one of the most important natural methods for wastewater treatment (Abbas *et al*., 2006) which are mainly shallow man-made basins comprising a single or several series of anaerobic, facultative or maturation ponds (Mbwele, 2006) which discharge a well-treated effluent after a retention time of many days (Mara *et al*., 1992). It is particularly well suited for tropical and subtropical countries because the intensity of the sunlight and temperature are key factors for the efficiency of the removal processes (Mara, 2001). Saqqar and Pescod (1995) recorded BOD, COD and SS removal efficiencies of 53%, 53% and 74% respectively and performances increased with an increase in temperature. Waste stabilization pond technology is the most cost-effective wastewater treatment technology for the removal of pathogenic micro-organisms which is achieved through natural disinfection mechanisms (Mara *et al*., 1992). WSPs have been classified according to the availability of oxygen for the stabilization process as anaerobic, facultative and aerobic (Mara *et al*., 1992; Hosetti and Frost, 1998; Bitton, 2005). There are three types of ponds; anaerobic ponds, facultative ponds and maturation/oxidation ponds. In essence, anaerobic and facultative ponds are designed for BOD removal and maturation ponds for pathogen removal, although some BOD removal occurs in maturation ponds and some pathogen removal in anaerobic and facultative ponds too. In many instances only anaerobic and facultative ponds are required whereas, maturation ponds are required only when stronger wastewaters (BOD > 150 mg L^{-1}) are to be treated prior to surface water discharge and when the treated wastewater is to be used for unrestricted irrigation (irrigation for vegetable crops) (Mara, 2001).

2.3.1.1 Anaerobic Ponds

Anaerobic ponds are deep treatment ponds that exclude oxygen and encourage the growth of bacteria, which further break down the effluent in the absence of oxygen "anaerobically" and releasing methane and carbon dioxide (Bitton, 2005). The anaerobic pond acts like an uncovered septic tank where sludge is deposited on the bottom and a crust forms on the surface (Fig. 1).

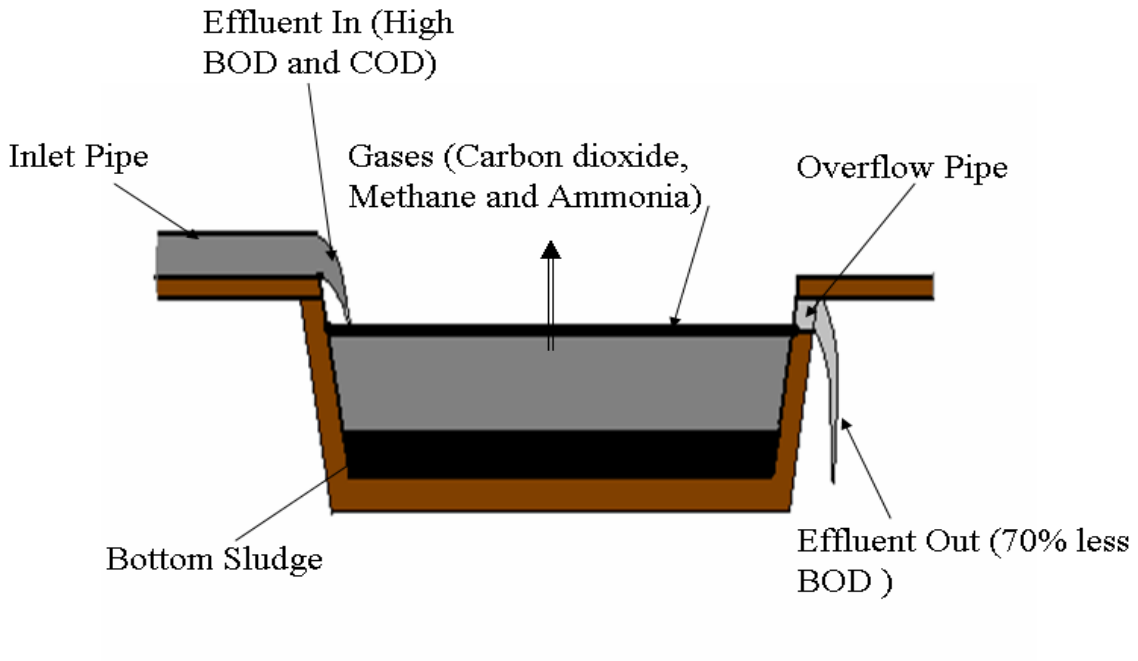

Fig. 1. Anaerobic pond (Ramadan and Ponce, 1998)

Anaerobic ponds are commonly 2-5 m deep and receive such a high organic loading (usually > 100 g BOD m^{-3} d^{-1} equivalent to > 3000 kg ha^{-1} d^{-1} for a depth of 3 m). They work extremely well in warm climate (can attain 60-85% BOD removal) and have relatively short retention time (for BOD of up to 300 mg L^{-1}, one day is sufficient at temperature > 20^{o}C) (Hosetti and Frost, 1998). Normally, a single anaerobic pond in each treatment train is sufficient if the strength of the influent wastewater is less than 1000 mg L^{-1} BOD_5. For high strength industrial wastes, up to three anaerobic ponds in series might be justifiable but the retention time in any of these ponds should not be less than 1 day (McGarry and Pescod, 1970). Designers have been in the past too afraid to incorporate anaerobic ponds in case they cause odor which is due to hydrogen sulphide (H_2S) produced under anaerobic conditions however, in the case of typical municipal sewage, it is generally accepted that a maximum anaerobic pond loading of 300 g BOD_5 m^{-3} d^{-1} at 20°C will prevent odor nuisance (Mara *et al*., 1992). However, results obtained from a more recent study in northern Brazil carried out by Pearson *et al*., (1996) suggest that maximum design volumetric loadings may increase to 350 g BOD_5 $m^{-3}d^{-1}$ at 25°C. Anaerobic ponds reduce N, P, K and pathogenic microorganisms by sludge formation and the release of ammonia into the air. As a complete process, the anaerobic pond serves to:

- Separate out solid from dissolved material as solids settle as bottom sludge.
- Dissolve further organic material.

- Break down biodegradable organic material.
- Store undigested material and non-degradable solids as bottom sludge.

2.3.1.2 Facultative ponds

Facultative ponds are 1-3 m deep which receive settled wastewater (usually the effluent from anaerobic ponds) and they are designed for BOD removal on the basis of a relatively low surface loading (100-400 kg BOD $ha^{-1}d^{-1}$ at temperature between 20°C and 25°C (Mbwele, 2006). The temperature conditions promote development of a healthy algal population as the oxygen for BOD removal by the pond bacteria is mostly generated by algal photosynthesis. Microalgae play an important role in system since they supply molecular oxygen to heterotrophic partners and thus support the initial steps of degradation (Cerniglia, 1992). Effluent entering the facultative pond from the anaerobic pond is converted into carbon dioxide, water and new bacterial and algae cells in the presence of oxygen. The activity of further anaerobic oxidation and the aerobic conversion of effluent to carbon dioxide, water and new bacterial and algae cells can result in removal of 80% of the BOD_5 of the effluent flowing into the facultative pond (which means an overall removal in the order of 95% over the two ponds). Moreover, as a result of the algal-bacterial activities, a high proportion of the BOD that does not leave the pond as methane ends up as algal cells. Thus in secondary facultative ponds (and in the upper layers of primary facultative ponds) "sewage BOD" is converted into "algal BOD" and this has important implications for effluent quality requirements. This provides even better BOD quality of the effluent from a facultative ponds as most of the BOD contained (70 to 90%) will be "algal BOD". Abis (2002) reported a BOD removal in pilot-scale facultative ponds in the United Kingdom (surface loading 51-117 kg ha^{-1} d^{-1}) to an average of 91% (between 67.5% and 98.6%). These values include the contribution of algae in the effluent. With the algal (and other) solids removed from the effluent, the average removal was 97.2% (with a range of 89.7-99.7%). Protozoan cysts and helminth eggs are removed by sedimentation. Their settling velocities are quite high (for example, 3.4 $x10^{-4}$ m s^{-1} in the case of *Ascaris lumbricoides)*, and consequently most removal takes place in the anaerobic and facultative ponds. It has recently become possible to design WSP for helminth egg removal (Ayres *et al*., 1992). In facultative and maturation ponds, ammonia is incorporated into new algal biomass. Eventually the algae become moribund and settle to the bottom of the pond; around 20% of the algal cell mass is non-biodegradable and the nitrogen associated with this fraction remains immobilized in the pond sediment.

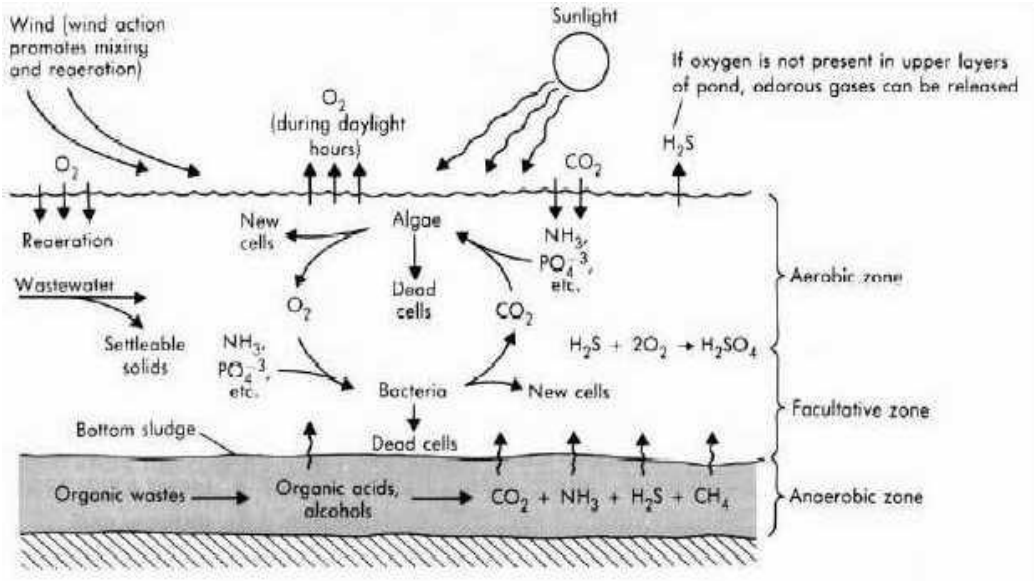

Fig. 2. Operation of the facultative pond (Tchobanoglous and Schroeder, 1985).

That associated with the biodegradable fraction eventually diffuses back into the pond liquid and is recycled back into algal cells to start the process again. At high pH, some of the ammonia will leave the pond by volatilization. Mara and Pearson (1986) point out that under certain conditions some algal species are able to adapt to and withstand concentrations of up to 50 mg L^{-1}. There is little evidence for nitrification (and hence denitrification, unless the wastewater is high in nitrates). The populations of nitrifying bacteria are very low in WSP due primarily to the absence of physical attachment sites in the aerobic zone, although inhibition by the pond algae may also occur. Total nitrogen removal in WSP systems can reach 80% or more, and ammonia removal can be as high as 95%. The efficiency of total phosphorus removal in WSP depends on how much leaves the pond water column and enters the pond sediments. This occurs due to sedimentation as organic P in the algal biomass and precipitation as inorganic P (principally as hydroxyapatite at pH levels above 9.5), compared to the quantity that returns through mineralization and resolubilization. As with nitrogen, the phosphorus associated with the non-biodegradable fraction of the algal cells remains in the sediments. Polprasert and Charnpratheep (1989) and Kaplan and his co-workers (1987) examined the fate of heavy metals in such ponds. Adsorption of metals was increased in attached-growth stabilization pond as compared to stabilization ponds without attached-growth. Kaplan and his co-workers (1987) reports only a slight decrease in total metals concentration, however the particulate fraction was mostly solubilized. A study by Moshe (1972) showed that high

concentrations of metal ions (Cd, Cu, Ni, Zn, and Cr) are toxic to *Chlorella* species, the most common species in stabilization ponds, and adversely affect pond efficiency. However, high pH (higher than 8) causes metal ions to precipitate and allows pond purification processes to occur normally. Many techniques have been developed to remove the algae from effluents; these include rock filtration, grass plots, floating macrophytes and herbivorous fish. Also, the use of maturation ponds can reduce the algal concentration considerably provided the system is not overloaded.

As a complete process, the facultative pond serves to:

- Aerobically break down most remaining organic solids near the pond surface.
- Reduce the amount of disease-causing microorganisms.
- Allow the loss of 20% to 30% of the ammonia, contained within the effluent, into the air.
- Store residues from digestion, as well as non-degradable solids, as bottom sludge.
- Allow treated effluent to pass out into a waterway or additional treatment system (i.e. an additional pond, wetland system or for land application).

2.3.1.3 Maturation ponds

Maturation ponds (low-cost polishing ponds, which succeed the primary or secondary facultative pond) are primarily designed for tertiary treatment, i.e., the removal of pathogens, nutrients and possibly algae. They are very shallow (usually around 1 m) ponds which allow light penetration to the bottom and aerobic conditions throughout the whole depth. The size and number of maturation ponds needed in series is determined by the required retention time to achieve a specified effluent pathogen concentration. In the absence of effluent limits for pathogens, maturation ponds act as a buffer for facultative pond failure and are useful for nutrient removal (Mara and Pearson, 1998). Mara and Pearson (1998) reported that if an anaerobic and secondary facultative pond system is used, this will produce an effluent suitable for restricted irrigation. Therefore, additional maturation ponds will only be needed if a higher quality effluent is required.

2.4 Additional technologies used to improve WSP effluent

The use of anaerobic and facultative ponds system, as the only wastewater treatment before final discharge, was proven to be satisfactory under different circumstances and for

various agricultural and aquacultural effluent reuses (Pearson *et al*., 1996; Mara, 2001). However, when some of the effluent quality limits are not satisfied, choosing a supplementary (or even alternative technology) in order to improve the effluent quality will be a serious option. The choice of adding new agents to the existing anaerobic and facultative ponds or choosing more advanced WSP treatment systems should be taken.

2.4.1 Integrated facultative ponds (Advanced facultative ponds)

One possible solution to benefit from the advantages of both anaerobic and aerobic ponds and suppress their disadvantages is to integrate the best functions of each pond type into a single pond to allow the symbiotic relationships of related microorganisms to proceed unrestrained. The advanced facultative pond is deep to promote sedimentation of wastewater solids and anaerobic decomposition of methane whereas, its most attractive feature is its high capability of wastewater total suspended solids (TSS) removal, in addition to BOD removal. The pond is designed so that its surface remains aerobic, thus reducing potential odor problem and biogas may be collected using submerged gas canopy and potentially used for energy production (Green *et al*., 1996).

2.4.2 Mechanical aeration

Aeration introduces oxygen to effluent standing in a facultative pond, so that bacteria can effectively convert the organic solids to carbon dioxide, water and bacteria biomass. Mechanically aerated ponds generate turbulence to mix all the effluent in the pond and introduce oxygen through equipment that either

- Introduces air into the effluent by injecting air under the pond surface (floating pumps).
- Exposes more effluent surface area to the air through spraying effluent into the air or agitating the effluent.

Aerator numbers and configuration are selected to perform the amount of oxygen generation needed. This technology can significantly reduce the nutrient, ammonia, odor, and BOD level in the resulted effluent.

2.4.3 Chemical treatment and biological additives

Several kinds of additives are available to control odors and break down crusting and organic matter. There are two distinct categories of additives used:

- Biological additives (bioremedation): Using bacteria, yeast and enzymes to degrade solids in ponds so that they are eventually liquefied. This may result in changes in BOD (may drop or may rise) and TSS (drop) concentrations and reduce temporary odor emission.
- Chemical additives: It is claimed that copper electrodes immersed in the pond reduce odors, kill pathogenic microorganisms and prevent build-up of crust.

2.4.4 Stabilization ponds and supporting growth media

In the pond modified by Zhao and Wang (1996), attached-growth media (AGM) or so-called artificial fibrous carriers were installed. This type of media consists of fine strings of polyvinyl acetate, with specific surface area of 1,236 $m^2 m^{-3}$ and cost only US$ 5 m^{-3}. A pilot-scale investigation has been conducted by them, using three ponds with working dimensions of 4.0 m in depth, 1.2 m in width and 1.1 m in depth. This study has confirmed that the incorporation of AGM enhanced the performance of conventional WSPs by formation of a great number of small stable ecological systems around AGM, being abundant in bio-species from bacteria and algae to protozoa, increasing the biomass concentration, improving the biological distribution. Better removal efficiencies of COD (75.6%), BOD (90.2%) and NH_4-N (68.5%) had been achieved in the WSPs with AGM than in the conventional WSPs, although the total retention time had been shortened to 7.5 days.

2.4.5 Advanced integrated wastewater pond system

Developed by Professor William J. Oswald and his co-workers at the University of California, Berkeley over the past four decades wastewater treatment and algae production systems called Advanced Integrated Wastewater Pond Systems (AIWPS) are potentially feasible for application in the developing world (Oswald, 1990). Although AIWPS may appear to be an adapted traditional pond system, each AIWPS facility is uniquely designed and incorporates a series of low-cost ponds or earthwork reactors. Depending on specific

effluent characteristics, regulatory requirements, human resources, and local climatic conditions, a typical AIWPS facility consists of at least four ponds in series.

- An advanced facultative pond with fermentations pits;
- Algal high rate Pond where photosynthetic oxygenation, oxidation, and nutrient assimilation occurs (with pedal wheel).
- Algal settling ponds; and
- A maturation pond where final effluent storage and further natural disinfection occurs.

AIWPS facilities are designed to minimize the accumulation of sludge and to maximize the production of oxygen through algal photosynthesis. Algal biomass is produced and can be used as a nitrogen-rich fertilizer, or as protein-rich animal or fish feed (for further cultivation of high protein foodstuffs), modern medicine and even cosmetics for the idle. They are cost-effective, require little maintenance and have generally performed well in terms of BOD_5 and solids removal. Moreover, AIWPS require similar land area to conventional lagoons, virtually eliminate sludge disposal, produce less odor, and may be adapted to energy (methane) recovery.

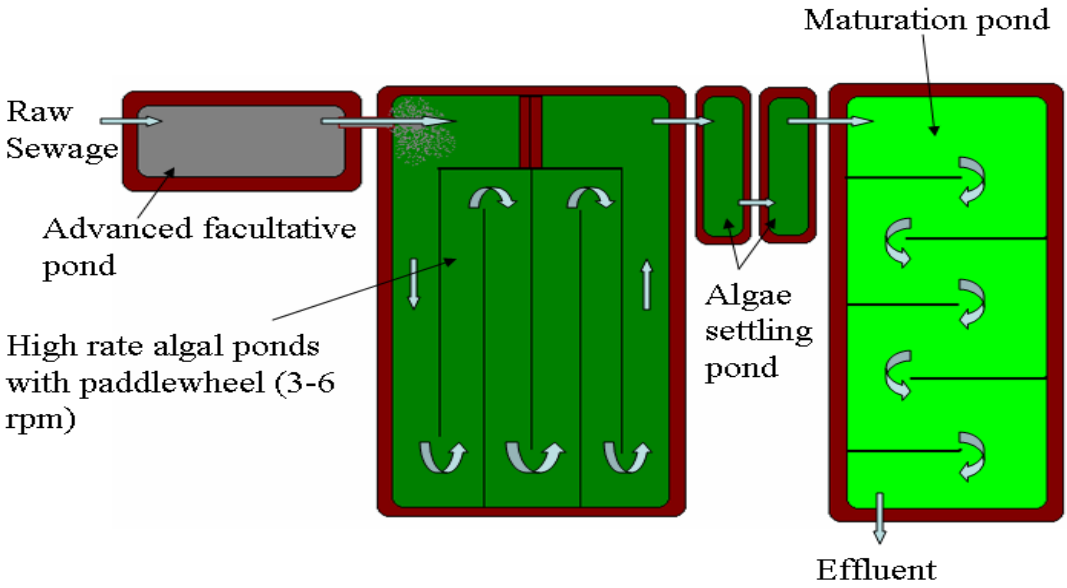

Fig. 3. Advanced integrated wastewater pond system (AIWPS) (Ramadan and Ponce, 1998)

2.4.6 Sheaffer modular reclamation and reuse system (SMRRS)

The Sheaffer system is described as a Modular Reclamation and Reuse System producing no sludge, no odor, and enabling 100% recovery of nutrient rich water for irrigation. The system is comprised of a deep aerated treatment cell, a storage cell, and three moving parts, described as a grinder pump, a compressor/blower, and an irrigation system (Ramadan and Ponce, 1998). The first stage of the process uses the grinder pump to reduce sewage solids influent and injects it to an anaerobic zone at the bottom of the treatment cell where it undergoes anaerobic reduction for a 14- to 30-day period. This zone acts as a mesophilic reactor. Solids settle out of the anaerobic zone to the base of the deep cell, and are stored for a time period of 20 to 30 years. The second stage of the process, the compressor/blower, injects air into the treatment cell just above the anaerobic zone to create aerobic conditions at the surface level of the cell. The cells are designed to provide 14- to 36-day treatment and further reductions of organic materials (Ramadan and Ponce, 1998). Solid components are broken down into simple organic acids, methane carbon dioxide, sulphide, ammonia, inorganic compounds, and water. The nitrogen, phosphorus, and potassium are dissolved and remain in solution for use in agricultural irrigation.

2.4.7 Aerated ponds/lagoons

A number of facultative ponds have been designed, or more commonly retrofitted, with surface aerators to boost dissolved oxygen levels and/or to aid mixing. There is often confusion between these systems and what are typically called aerated lagoons. Unlike facultative ponds, aerated lagoons are designed to operate at high bacterial cell mass concentrations. These require a high power input for aeration and in some cases incorporate biomass return. They operate at much shorter hydraulic residence times and as a consequence of this, and their increased depth, do not develop significant algal populations. Aerated lagoons are essentially designed to work as a form of lowly loaded activated sludge. Mechanically supplied oxygen increases treatment efficiency and reduces land requirements. However, the high-cost power input is sufficient only for diffusing oxygen into the pond and not for mixing the contents.

2.4.8 High-rate algal ponds

Originally developed by Oswald at the University of California in the sixties, high-rate algal ponds have continued to be developed and implemented particularly in the United

States. These systems are shallower than a facultative pond and operate at shorter hydraulic retention times. A paddlewheel is normally incorporated to drive the water around a "race-track" shaped pond. The oxygen production is reported to be significantly higher than typical facultative pond designs. The micro algae produced in these systems are also reported to have good settling properties (Green *et al*., 1996).

2.4.9 Rock filters

Waste stabilization ponds often have high concentrations of TSS in the effluent, which may or may not be desirable depending on the irrigation delivery method. Middlebrooks (1995) suggests that many low-cost methods exist for polishing WSP effluent, which include intermittent sand filtration and rock filters. Rock filters, when used in conjunction with WSPs, have been shown to upgrade WSP effluent. Research at a pilot-scale rock filter demonstration conducted at the Assamra WSPs in Jordan showed that effluent content reductions could be reduced greatly. TSS and BOD were reduced by 60%, total faecal coliform count (TFCC) by a maximum of 94% and T-P by 46% at a loading rate of 0.33-0.044 kg m^{-3} of TSS (Saidam *et al*., 1995).

2.4.10 Microalgae and duckweed-based pond systems

Worldwide, there is continuous interest in algae and duckweed based pond systems that are inexpensive and are known for their ability to achieve good removal of pathogens and organic pollutants. The feasibility of the microalgae and duckweed based pond systems for domestic wastewater treatment has been documented in the literature (Reddy and DuBusk, 1985; Alearts *et al*., 1996). Microalga and duckweed systems are one of the options that have been widely applied for combined handling of wastewater with the nutrients used for poultry and aquacultural projects (Ramadan and Ponce, 1998; Nhapi *et al*., 2001). It has been applied at full scale in Taiwan, China, Bangladesh, Belgium and USA (Reddy and DeBusk, 1985).

Microalgae are excellent for nutrient removal processes, as they exhibit high contents of N and P, about 10 and 1% respectively on a dry weight basis, several-fold that of higher plants. Also, microalgae cultures are able to reduce residual concentrations of these nutrients to vanishingly low levels and allow for a significant variability in N:P ratios. The removal of heavy metals from wastewaters has been extensively studied and some actual applications with immobilized algae were reported, though these could not compete commercially with ion exchange resins (Wilde and Benemann, 1993). However,

high algal concentration of about 100 mg TSS L^{-1} may occasionally reached in the effluent (Middlebrooks, 1995), causing severe clogging problems in advanced irrigation system (Pearson *et al*., 1995). Microalgae and duckweed-based pond system generate biomass that is known to be an excellent source of feed for fish or poultry raising (Oron, 1994) and yield good effluent quality for irrigation. The pollutant removal efficiency of such pond systems varies widely depending on retention time, water depth, initial nutrient concentration, duckweed density, used genera type, and harvesting regimes. Some figures are: Nitrogen 34 to 99%, phosphorus 12 to 92%, and BOD_5 65 to 90% (Oron *et al*., 1984; Ramadan and Ponce, 1998).

Table 1. Overview of achievable effluent qualities for natural systems (Ramadan and Ponce, 1998).

Natural systems	*Hydraulic retention time (days)*	*BOD (mg L^{-1})*	*TSS (mg L^{-1})*	*Total N (mg L^{-1})*	*Faecal coliforms (no. 100 ml^{-1})*	**Reference**
High rate anaerobic reactor (pre-treated)	0.25	22-80	30-105	20-85	10^5-10^7	Lettinga *et al*., 1993
Waste stabilization ponds (AP + FP)	10-40	20-40	80-120	-	-	Reed *et al*., 1995
Waste stabilization ponds (AP+FP+MP)	14-23	-	-	0-20	<100	Silva *et al*., 1995; Arridge *et al*., 1995
Duckweed ponds	14-28	5-40	40-80	3-45	10^2-10^4	Zimmo *et al*., 2002
Constructed Wetlands	3-15	5-40	5-20	5-10	10^2-10^5	Reed *et al*., 1995

In terms of duckweed systems, there would be problems with ammonia poisoning, as duckweed requires about 20 to 60 mg ammonia to grow actively. Above this range, a toxic effect has been reported due to high levels of free ammonia in the water (Das, 1998; Kyoburungi, 1998; Caicedo *et al*., 2000). Algae and duckweed grows on the surface of ponds so its yield can be optimised by providing optimal growth area but this will also be governed by harvesting mechanisms (Iqbal, 1999). Floating booms dividing the pond into about 50 m^2 bays could also be used to avoid wind effect and easier access for harvesting.

However, from a sustainable nutrient management point of view, the pond system effluent should be further usefully applied for other purposes like fish farming and crop irrigation (Ramadan and Ponce, 1998). Nhapi and his co-workers (2003) recommended a pond system design which is flexible enough to change over to waste stabilisation pond systems when adverse conditions in influent quality are obtained. In some cases, this would require the inclusion of a parallel pair of anaerobic ponds as a form of pretreatment. Although natural systems are prone to poisoning by industrial effluents, microalgae and duckweeds are known to tolerate and accumulate heavy metals (Iqbal, 1999) whereas, experiments have been carried out on chromium removal using duckweed and microalgae and these had positive results (Sultan, 1999). Like any other wastewater treatment system, algal and duckweed based systems need constant monitoring. Because of longer response and retention times, monitoring of parameters like TKN, NH_4^+, NO_3, COD, temperature, pH and DO should be done monthly. Nhapi *et al*., (2003) actually concluded that higher removal potentials exist if due attention is paid to design and operational criteria.

2.5. Algal mediated removal of heavy metals in pond system

A considerable amount of research has been conducted on algae as a bioremediation agent, but this research has primarily focused on the ability of some species to adsorb or accumulate metal ions from solution (Harris and Ramelow, 1990). Little research has targeted the fate of heavy metals in WSP and HRAP (Kaplan *et al*., 1987; Toumi *et al*., 2000). The production of extra cellular components, such as chelating with polysaccharides, which are capable of complexing metal ions has also been extensively studied (McKnight and Morel, 1980). While the ability of certain algal species to increase the alkalinity of the surrounding medium as a by-product of their inorganic carbon accumulating mechanism has been documented (Shiraiwa *et al*., 1993), the potential use of this alkalinity in the precipitation of metals has, to date, not been widely reported.

Heavy metals may be removed (Table 2) by a variety of processes in WSPs. The main heavy metal removal process include

1. Sedimentation of wastewater solids
2. Adsorption of algal/bacterial biomass and bottom sludge
3. Bioaccumulation into algal/bacterial biomass
4. Chelation
5. Precipitation

Domestic wastewater fed into WSPs often contains elevated concentration of lead, cadmium, chromium, copper and zinc due to corrosion of water pipes and plumbing. Uptake (adsorption and bioaccumulation) by pond algae has been shown to be an important heavy metal removal process (Soniassy and Lemon, 1986) which was followed by these algae and bacteria sedimentation (Shilton, 2006). Most removal occurs in primary ponds (anaerobic or facultative) and is due to sedimentation of solid to which the heavy metals have adsorbed (Nejmeddine *et al*., 2000, Toumi *et al*., 2000). The involvement of sedimentation as dominating mechanism in heavy metal removal was supported by work of Nejmeddine and his co-workers (2000) where anaerobic stabilization pond reduced the load of Zn, Cu and Pb with respective efficiencies of 28%, 21% and 11%. Heavy metals, zinc (Zn), copper (Cu) and lead (Pb) removal in waste stabilization pond (WSP) and high rate algal pond (HRAP) units were compared where the removal rate for the three elements reached 91, 92 and 71% respectively for Zn, Cu and Pb in the WSP train and 89, 88 and 51% for HRAP (Table 2). However, since the two trains were not receiving the same flow, the use of specific removal rate expressed as mg removed m^{-2} d^{-1} showed that the HRAP was 1.3, 10 and 2 times more efficient respectively for Zn, Cu and Pb removal than the three facultative ponds in series of the WSP (Toumi *et al*., 2000). In similar study elimination rates of 80%, 87%, 38%, 60% and 63%, respectively for Zn, Cu, Pb, Cd and Cr was reported by two phase anaerobic reactor (RAP) - high rate algal pond (HRAP) system. However, the essential metal removal was ensured by the HRAP and the two maturation ponds whereas, primary treatment (RAP) was not involved in this reduction.

Further research is required on ways to enhance heavy metal uptake by pond algae and bacteria and potentially use them to mine heavy metals from wastewater. Moreover research is needed on reducing the release of heavy metals from pond sludge back to the pond water.

2.6. Microalgae in metal removal

Metal pollution is a global concern and the levels of metals in all environments, water, air and soil, are increasing, in some cases to toxic levels, with contribution from a wide variety of industrial and domestic sources (Mehta and Gaur, 2005). Discharge of heavy metals to the environment not only results in acute toxicity to aquatic organisms, but also has longer term effects through the bioaccumulation and biomagnification in aquatic community (Shilton, 2006). A number of physicochemical methods, such as chemical precipitation, adsorption, solvent extraction, ion exchange, membrane separation, etc.,

have been commonly employed for stripping toxic metals from wastewaters (Eccles, 1999). However, these methods have several disadvantages, such as incomplete metal removal, expensive equipment and monitoring system requirements, high reagent or energy requirements and generation of toxic sludge or other waste products that require disposal. Further, they may be ineffective or extremely expensive when metal concentration in wastewater is in the range 10–100 mg L^{-1} (Mehta and Gaur, 2005).

Table 2. Metal removal ability of aquatic systems.

Water Treatment System	Heavy Metal	% Removal	Reference
WSP algal culture	Cu	70-90	Filip *et al*., 1979
	Cd	70-90	
	Cr	20	
Facultative Ponds	Cu	60	Soniassy and Lemon, 1986
	Cd	90	
	Hg	70	
	Ni	99	
	Pb	83	
	Zn	90	
Anaerobic Pond	Cu	21	Nejmeddine *et al*., 2000
	Pb	11	
	Zn	28	
Wastewater stabilization pond (AP,3 FP, 2 MP)	Cu	92	Toumi *et al*., 2000
	Pb	71	
	Zn	91	

In recent years, there has been increasing interest in the use of biomass from microbial sources, particularly the microalgae to absorb heavy metal ions (Table 3) as part of remediation efforts (Dönmez and Aksu, 2002; Davis *et al*., 2003; Chojnacka *et al*., 2004). These microalgae (phytoplankton) in the oceans live in an environment which comprises more than 70% of the earth's surface and is responsible for at least 32% of global photosynthesis (Whittaker, 1975). Through 500 million years of evolution and within this large competitive environment, these microalgae have developed a myriad of polymers which can scavenge the metals of interest, many of which are essential nutrients

utilized at low concentrations in microalgal metabolism. In fact, these polymeric materials in the particulate fraction of the world's oceans and lakes regulate the distribution of heavy metal ions in natural waters.

Table 3. Metal sorption capacity of various algae.

Algae	Metal	Sorption (mg g^{-1})	Reference
Chlorella vulgaris	Zn	6.60	Sandau *et al*., 1996
Spirulina sp.		0.20	Chojnacka *et al*., 2004
Microcystis sp.		999.50	Pradhan *et al*., 1998
Laminaria japonica		56.87	Lee *et al*., 2004
Lyngbya taylorii		32.03	Klimmek *et al*., 2001
Ulvafasciata sp.		13.5	Prasanna *et al*., 2007
C. vulgaris	Pb	17.2	Sandau *et al*., 1996
Laminaria Japonica		349.09	Lee *et al*., 2004
Spirulina platensis		16.98	Sandau *et al*., 1996
Cladophora fascicularis		198.5	Deng *et al*., 2007
Spirogyra neglecta		116.1	Singh *et al*., 2006
Ascophyllum sp.	Cr^{3+}	129.99	Kratochvil and Volesky, 1998
Sargassum sp.		68.89	Cossich *et al*., 2002
Spirulina sp.		9.62	Chojnacka *et al*., 2005
Gelidium sp.		18	Vilar *et al*., 2007
Padina sp.	Cr^{6+}	54.60	Sheng *et al*., 2005
Sargassum sp.		31.72	Sheng *et al*., 2005
Gracilaria salicornia		45.959	Khorramabdi and Soltani, 2008
Sargassum sp.		33.258	Khorramabdi and Soltani, 2008
C. vulgaris	Ni	205.48	Mehta and Gaur, 2001a
C. vulgaris (Acid pretreated)		437.98	Mehta *et al*., 2002a
Microcystis aeruginosa		249.98	Pradhan *et al*., 1998
Sargassum sp.		180.83	Kalyani *et al*., 2004
C. vulgaris	Cu	190.62	Mehta and Gaur, 2001b
Laminaria japonica	Cd	146.12	Yin *et al*., 2001

Microalgae are so efficient at scavenging of metals from influent water, from contaminants in nutrients, or from atmospheric deposition into open ponds, that the biomass produced sometimes can contain amounts at the upper limit of metal content for food use (Kajan *et al*., 1992). It should be pointed out that the concentrations of the required elements for microalgae in the natural environment are very low, and are sometimes responsible for the dominance of one phytoplankton group over another (Wetzel, 1983). For instance, copper is often present at only 1- 2 ppb in lakes, yet is a required nutrient (Wetzel, 1983). A significant bonus is that uptake in the natural environment typically proceeds in the presence of high concentration of divalent (Ca^{2+}, Mg^{2+}) or monovalent (Na^{+}, K^{+}) cations in both freshwater and marine environments.

These microalgae can be cultivated inexpensively as biomass (Brown and Zeiler, 1993) and provide a ready source of these complex polymeric mixtures where the wide range of functional groups present on macromolecules, including polysaccharides, proteins, peptides and nucleic acids which accounts for the majority of metal adsorption by ion-exchange mechanisms (Romero-Gonzalez, 2001; Skowron˜ski and Ska, 2000; Mehta and Gaur, 2005). The accumulation of heavy metals in algae involves two processes: an initial metabolically independent (passive) adsorption where metal ions adsorb onto the cell surface followed by a much slower metabolism-dependent (active) uptake which transport of metal ions across the cell membrane into the cytoplasm (Bates *et al*., 1982). Adsorption was found to contribute >80 % than uptake by Mehta and his co-workers in 2002a whereas in few previous reports it has been suggested a greater contribution of uptake than adsorption to total metal accumulation (Avery *et al*., 1998). Adsorption onto the cell surface (wall, membrane or external polysaccharides) involve negatively charged hydroxyl (OH), phosphoryl (PO_3O_2), amino (NH_2), carboxyl (COOH), sulphydryl (SH) functional groups which dissociate into corresponding anion and proton at a specific pH where anion binds cationic metal ions in water (Niu and Volesky, 2000; Skowron˜ski and Ska, 2000; Romero-Gonzalez *et al*., 2001). The distribution and abundance of cell wall components and so as the kinds of functional group vary among different algal groups e.g. carboxyl group in cyanobacteria cell wall plays an important role in Cu, Cd and Pb binding whereas its role in Au (III) binding was documented for *Chlorella vulgaris* (Kuyucak and Volesky, 1989; Yee *et al*., 2004; Chojnacka *et al*., 2005). Similarly Gong and his co-workers (2005) concluded that amino and hydroxyl groups play an important role in Pb binding on *Spirulina maxima*. The advantage of anionic thick capsule of cyanobacteria in binding of heavy metals over thin capsulated counterparts was

well reported by De Philippis and his co-workers (2003). The excellent metal binding capacity of alginate as a main constituent of brown algae, mainly isolated from marine water has been widely acknowledged (Kuyucak and Volesky, 1989).

Recent studies reveal that the process of heavy metal biosorption involves four mechanisms

1. Ion exchange
2. Complexation or coordination
3. Electrostatic attraction
4. Microprecipitation

Ion exchange has been shown to be the most important mechanism for the biosorption of metal ions by algal biomass where unicellular algae show higher ion exchange capacity than filamentous forms due to higher surface/volume ratio (Crist *et al*., 1990; Pirszel *et al*., 1995) whereas Raiz and his co-workers (2004) supported the dominating role of complexation mechanism for metal sorption by algae. Electrostatic attraction and covalent binding, respectively, mediate Ni and Zn adsorption on *Chaetophora elegans* (Andrade *et al*., 2005) whereas, once metals are inside the cell, they may bind to intracellular components or precipitate with metal binding proteins, polyphosphate bodies where they may be detoxified (Gadd, 1988; Zhang and Majidi, 1994).

Extensive study have been carried out to explore live microalgae in metal removal whereas limited efforts have been made to use dead algal biomass for removing metal ions from wastewater even their metal removal ability at times higher than that of other commercially used sorbents (Table 1). Microalgal biomass showed an another comparative advantage of removing metals at very low concentration < 10 mg L^{-1} where commercial bioresins are incapable (Mehta *et al*., 2002). The findings showed that Pb was maximally sorbed by algal species probably due to its greater electronegativity and smaller ionic radii (Davis *et al*., 2003). Biosorption was found to be effected by various factors like external metal concentration, pH, biomass concentration, temperature and presence of other cations and anions. Biosorption increased initially by increasing initial metal concentration (Mehta *et al*., 2002a) and become saturated after certain concentration whereas, pH needs to be optimized for a metal ions against specific microalgae due to variable nature of their chemical interactions with algal cells. In general acidic pH (3–5) is most favorable for the sorption of metal ions. Pb was maximally sorbed at pH 5.0, when cell wall is negatively charged (Aksu and Kutsal, 1991) whereas Cr^{6+} on the surface that favors the binding of Pb^{2+} to surface ligands whereas Cr^{6+} is anionic in nature and bind at

lower pH when algal surface is positively charged (Aksu and Acikel, 1999). It has also been shown that increasing biomass of algae resulted in decrease in metal binding per unit biomass as increase in *Spirulina maxima* from 0.1 to 20 mg L^{-1} resulted in decrease of sorption potential from 121 to 21 mg g^{-1} (Gong *et al*., 2005) whereas similar observations were also reported for Cr, Co, Ni and Cd by four different algae (Hamdy, 2000). The change in temperature was found to be affecting metal uptake by live algal cells due to the involvement of various cell metabolisms however the effect of temperature was not as pronounced for biosorption (Cossich *et al*., 2002). Metal sorption of U, Co and Cu was inhibited by presence of anions e.g. carbonates, sulphate whereas light cations found in most industrial effluent with heavy metals also showed inhibitory effect to biosorption attributed to competition for cellular binding sites (Rai *et al*., 1981; Low *et al*., 2000). The other compounds that could be considered as impurities in metal removal are surfactants and EDTA as chelating agents (Kaplan *et al*., 1995).

However, the fragile nature of algale is not suitable for robust wastewater treatment operations (Chu *et al*., 1997), which has led to the interest in the use of entrapped biomass as immobilized preparations. Immobilized biomass within or on inert matrix has the inherent advantage that high flow rate can be achieved with minimal clogging of the column and constant size of sorbent particle and high biomass loading of the column reactor are feasible (Yakubu and Dudeney, 1986). Immobilization is a general term that describes many different forms of cell attachment or entrapment on polymer either natural, including agar, alginate and carrageenan, or synthetic, such as silica gel and polyacrylamide (Lopez *et al*., 1997).

Among synthetic polymers, polyacrylamide has been most extensively used where Akhtar and his co-workers developed a new biosorbent by immobilizing *Chlorella* within luffa sponge discs (Robinson and Wilkinson, 1994; Akhtar *et al*., 2003). Darnall and his co-workers in 1986 reported their results using silica to immobilize algal cells for use in "AlgaSORB" columns that are employed in remediation of industrial streams (Darnall *et al*., 1986). Silica gel has been extensively used for immobilization of algal cells or biomass (Rangasyatorn *et al*., 2004). Beside slow flow rate, immobilization on silica could offer some advantages such as improved mechanical strength and chemical stability which could lead to a new approach of immobilizing algae for industrial wastewater to meet the need of removal of metals in large quantity.

MATERIALS AND METHODS

1. Instruments Used

Instruments used during the course of this work were Atomic absorption spectrophotometer (*GBC 932AA, Australia*); Shaker incubator (Shivaki, New Delhi); Cooling centrifuge (Sigma 1-15, Germany); Microfuge (Sigma 1-15, Germany), Microscope (Nikon Eclipse E200, Japan), Hot air oven (Oven Universal, Narang Scintific Works Pvt. Ltd., New Delhi), Autoclave (Equitron, Mumbai), BOD incubator (Narang Scintific Works Pvt. Ltd., New Delhi), Serological water bath (Narang Scintific Works Pvt. Ltd., New Delhi), Microwave (LG, Mumbai), Laminar air flow hood (Thermadyne, Faridabad), COD reactor (Hach Heating, USA).

2. Study site and collection of wastewater

The model object was a wastewater stabilization pond system at village Sanghol in tehsil Khamano located in Fatehgarh Sahib District of Punjab also known as Ucha Pind Sanghol with an approximate population 53,397 is located at a latitude of 30.7833, longitude 76.3833 and altitude (Feet) 843 which lie in time zone (est) of UTC+5:30. The day temperature ranges from 45°C in May -June to 4°C in December-January and has a sub-tropical continental monsoon climate with satisfactory rain fall. It is about 40 km from Chandigarh on the way to Ludhiana which holds a special position on the archaeological atlas of India as excavation at the site have yielded coins and seals related to Nomadic rulers.

The village demonstrates an effective way of treating domestic wastewater generated by local population. The village is situated on a height so the drainage of domestic wastewater produced by whole village is collected to a common place by sewage pipe lines and treated into a wastewater stabilization pond system using microalgae. The system constituted an anaerobic pond (Length = 6.55 m x Breadth = 3.54 m), a facultative pond (Length = 84m x Breadth = 53m x Depth = 1.22 m) and a maturation pond (Length = 84m x Breadth = 50m x Depth = 1.83 m) in series. The wastewater generated was applied directly to the anaerobic pond where the settling of sludge and anaerobic treatment takes place with the help of anaerobic bacteria. Anaerobically treated water was passed to Pond 1 which is a facultative pond where the microalgae and duckweeds utilise inorganic nutrient for their growth and produce nascent oxygen during photosynthesis and thus augments complete oxidation of organic compounds lowering COD and BOD_5. After a

residence time of 12-14 days the treated wastewater was passed to pond 2 which acted as a maturation pond in wastewater treatment system for final treatment of treated water.

The wastewater samples were collected after 30 days interval during August 2004 to May 2005 from inlet and outlet of facultative ponds in autoclaved plastic bottles, which were brought to the laboratory and characterized for Biochemical oxygen demand, Chemical oxygen demand, Total solids, Total suspended solids, Total dissolved solids and bacterial count. Parameters such as water temperature, conductivity, salinity and dissolved oxygen (DO) were recorded on-site using portable probes from Thermo Orion Model 150 and 125 respectively. Wastewater samples were also screened microscopically for the microalgal diversity present and its comparative dominance.

3. Wastewater Analysis

3.1 pH

The pH of wastewater samples were measured using portable water testing kit (Thermo Orion model 290, USA). For this, the standard buffer solution of pH 4.1, 7.0 and 9.18 were prepared to calibrate the electrode. This calibrated electrode then rinsed with distilled water, wiped to dry the electrode and immersed into the sample to read the pH.

3.2 Electrical conductivity and salinity

Electrical conductivity and salinity were measured using a portable water testing kit (Thermo Orion model 125, USA). 100 ml of vigorously shaken sample was taken in a beaker and gave temperature compensation to the instrument. This electrode then rinsed with distilled water, wiped to dry the electrode and immersed into the sample to read electrical conductivity and salinity.

3.3 Determination of TS, TDS, and TSS

Total solids, Total suspended solids and Total dissolved solids were determined as per the method given by Cleseri *et al*., (1998)

Total solids

Procedure

1. 100 ml beakers were cleaned and heated to 103 to 105 °C for 1 hour and stored in desiccator to get constant initial weight of beakers.

2. The beakers were weighed immediately after removing from desiccator to get initial weight of each beaker.
3. 50 ml of well mixed wastewater sample was transferred to preweighed beakers and kept in drying oven at 98°C till complete evaporation of water.
4. Evaporated samples were again dried for one hour in an oven at 103 to 105 °C and cooled in desiccator to balance temperature and weight.
5. The beakers were weighed as soon as they had cooled down for final weight.

Calculation

$$\text{Total solids (mg L}^{-1}) = \frac{(A - B) \times 100}{sample\ volume\ (ml)}$$

Where:

A is the final weight of beaker representing weight of residue and beaker (mg).

B is initial weight of beaker (mg).

Total dissolved solids

The total dissolved solids were determined in wastewater following same procedure and calculations as used for determination of total solids; where as a well mixed sample was filtered using whatman No. 42 filter paper (Whatman Pvt. Ltd., Mumbai) before transferring 50 ml sample to 100 ml preweighed beakers.

Total suspended solids

A well mixed wastewaters contained total solids, which are sum of total dissolved solids and total suspended solids, so total dissolved solids were calculated using calculation as follows.

Calculation

Total suspended solids (mg L^{-1}) = Total solids (mg L^{-1}) - Total dissolved solids (mg L^{-1})

3.4 Biochemical oxygen demand

Biochemical oxygen demand of wastewater samples were determined as per the method given by Cleseri *et al*., (1998)

Reagents

1. Phosphate buffer solution: 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $NaHPO_4$ .7H2O, and 1.7 g NH_4Cl were dissolved in about 500 ml double distilled water and diluted to 1 L.
2. Magnesium sulphate solution: 22.5 g $MgSO_4.7H_2O$ was dissolved per 1 L of double distilled water.
3. Calcium chloride solution: 27.5 g $CaCl_2$ was dissolved in 1 L double distilled water.
4. Ferric chloride solution: 0.25 g $FeCl_3$.6H2O was dissolved in 1L double distilled water.
5. Sodium sulphite solution: 1.575 g Na_2SO_3 was dissolved in 1L double distilled water. Solution was freshly prepared at each time of analysis.
6. Glucose-glutamic acid solution: Glucose and Glutamate of analytical grade were dried in oven at 103 °C for 1 hour from which 150 mg of glucose and 150 mg glutamate were dissolved in 1 L of double distilled water.
7. Ammonium chloride solution: 1.15 g NH_4Cl was dissolved in about 500 ml double distilled water and pH was adjusted to 7.2 with 1N HCl and 1N NaOH solution, and final volume was made up to 1L with double distilled water.
8. Manganous sulphate solution: 480 g of $MnSO_4.4H_2O$, 400 g $MnSO_4.2H_2O$, were dissolved in double distilled water and filtered followed by volume made up to 1L with double distilled water.
9. Alkali-iodide-azide reagent: 500 g of NaOH and 135 g NaI were dissolved in 1L of double distilled water, followed by addition of 10 g NaN_3 previously dissolved in 40 ml double distilled water.
10. Sulfuric acid (H_2SO_4) Concentrated.
11. Starch: 2 g of laboratory-grade soluble starch and 0.2 g salicylic acid were dissolved in 100 ml of hot double distilled water.
12. Standard sodium thiosulphate: 6.205 g of $Na_2S_2O_3.5H_2O$ was dissolved in double distilled water followed by addition of 0.4 g solid NaOH and diluted to final volume of 1L.

Procedure

1. Dilution water was prepared by adding 1 ml L^{-1} of each phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions and saturating with dissolved oxygen by aerating with organic free filtered air for 4 hours.
2. 1ml previously acclimatized seed was also added to 1 L dilution water before use.
3. Wastewater sample was diluted appropriately with dilution water and filled in BOD bottles of 300 ml capacity by siphoning to avoid any air bubbles. The bottles were closed tightly with stopper and mixed well.
4. Initial DO of one bottle for each diluted sample, dilution water blank and standard (glucose-glutamic acid) was measured immediately after filling BOD bottles with azide modification method.
5. In azide modification method 1 ml of $MnSO_4$ solution was transferred to BOD bottle containing sample, followed by addition of 1 ml alkali-iodide-azide reagent with separate glass pipets alternatively by dipping them into the sample.
6. BOD bottles were tightened with stopper and mixed by inverting to form precipitates.
7. 1 ml of concentrated sulfuric acid was added to the solution holding pipet tip just above liquid surface after settling of precipitates to half of the bottle.
8. Bottles were restoppered and mixed by inverting several times until dissolution is complete.
9. 201 ml of this sample was taken in titration flask and titrated with 0.025M $Na_2S_2O_3$ using starch solution as indicator to an endpoint of pale straw colour.
10. Initial D.O. of sample was calculated using following equation
    For titration of 200 ml sample, 1 ml 0.025 M $Na_2S_2O_3$ = 1 mg DO L^{-1}
11. Continuing step 5, another bottle of each sample was tightly closed and incubated for 5 days at 20 $^{\circ}$C which was analysed after 5 days of incubation for DO of samples following step 5-10. A blank and standard were also analysed after 5 days.

Calculations

$$BOD\ (mg\ L^{-1}) = (S1-S2)-(B1-B2)\ F/P$$

Where:

S1= initial DO of diluted sample/ standard (mg L^{-1})

S2= final DO of diluted sample /standard after 5 days incubation at 20°C (mg L^{-1})

B1= initial DO of seed control (blank) (mg L^{-1})

B2= final DO of seed control after 5 days incubation at 20°C (mg L^{-1})

F= ratio of seed control (% seed in diluted sample)

P= decimal volumetric fraction of sample used

3.5 Chemical oxygen demand

Chemical oxygen demand of wastewater samples were determined as per the method given by Cleseri *et al*., (1998)

Reagents

1. Standard potassium dichromate solution: 12.259 g of previously dried $K_2Cr_2O_7$ of primary standard grade was dissolved in double distilled waster and diluted to 1L.
2. Sulfuric acid reagent: 5.5 g of Ag_2SO_4 of technical grade was added to 1 L of concentrated H_2SO_4 and kept for one to two days for complete solubilisation of Ag_2SO_4.
3. Ferroin indicator solution: Ferroin solution (o-Phenanthroline ferrous sulphate complex) was purchased from s. d. fine-chem. limited, Mumbai.
4. Standard ferrous ammonium sulphate (FAS) titrant (0.25 M): 98 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ was dissolved in double distilled water followed by addition of 20 ml concentrated H_2SO_4, cooled and final volume made up to 1L. This solution was standardized against standard $K_2Cr_2O_7$ solution before use.*

* Standard $K_2Cr_2O_7$ was diluted to 100 ml with double distilled water followed by addition of 30 ml concentrated H_2SO_4, cooled and titrated with FAS titrant using 2 to 3 drops of ferroin indicator.

$$Molarity\ of\ FAS\ solution = \frac{Volume\ \ 0.0417\ M\ K_2Cr_2O_7\ solution\ titrated\ \ (ml)}{volume\ of\ \ FAS\ used\ in\ titration\ \ (ml)} \times 0.25$$

5. Mercuric sulfate: $HgSO_4$ powder.
6. Potassium hydrogen phthalate (KHP) standard: 425 mg of previously dried (102°C) potassium hydrogen phthalate ($HCOOCC_6H_4COOK$) was diluted to 1L by

double distilled water. This solution is stable and has theoretical COD of 1.176 mg O_2 mg^{-1}.

Procedure

1. Open reflux method was used to analyse chemical oxygen demand of wastewater samples.
2. 50 ml of appropriately diluted wastewater samples were suspended into 500 ml round bottom flasks of 500 ml followed by addition of 1 gm mercuric sulphate. Glass beads measuring 0.4 mm were added to prevent bumping.
3. 5 ml of concentrated sulfuric acid was added to reaction mixture very slowly and by proper mixing of solution to dissolve $HgSO_4$. The mixture was also cooled by water bath while mixing to avoid any possible loss to volatile materials.
4. 25 ml of 0.0417 M $K_2Cr_2O_7$ was also added to the solution followed by addition of remaining 70 ml of concentrated H_2SO_4 while continuing swirling and mixing of solution.
5. The solution was digested in digestion unit for 2 hr.
6. The digested solution was titrated against 0.25 M ferrous ammonium sulphate (FAS) using two to 3 drops of ferroin indicator to an end point of sharp colour change from blue-green to reddish brown.

Calculation

$$\text{COD as mg } O_2 \text{ } L^{-1} = [(A-B) \times M \times 8000] / \text{ml sample}$$

Where:

A = ml FAS used in blank,

B = ml FAS used in sample,

M= Molarity of FAS,

8000 is milli equivalent weight of oxygen

3.6 Culturable bacterial enumeration

Bacterial counts were carried out according to the standard plate count method on Nutrient agar plates (Cappuccino, 2004).

Nutrient Agar (g L^{-1})

Peptone	5.0g
Sodium chloride	5.0g

Beef extract	1.5g
Yeast extract	1.5g
Agar	15 g
pH	7.0

Procedure

1. Representative 1 ml of wastewater samples was added to a test tube containing 9 ml sterile water making a dilution corresponding to 10^{-1}.
2. Further dilutions were prepared in a similar way u p to 10^{-6}.
3. 100 µl of appropriate dilutions was added to the petri plate containing solidified nutrient agar and the inoculum was spread with the help of a sterilized spreader
4. The plates containing inoculum were incubated at 32°C for 24 hours and colony forming units were counted.

4. Screening of microalgae from wastewater

4.1 Algal culture media and culture conditions

BG-11 media

BG-11 media was prepared as per the composition given by Stanier *et al*., (1971).

Composition

Sodium nitrate ($NaNO_3$) 1.5g L^{-1}*; Di potassium hydrogen phosphate (K_2HPO_4) 0.04g L^{-1}; Magnesium Sulphate ($MgSO_4.7H_2O$) 0.075g L^{-1}; Calcium Chloride ($CaCl_2.2H_2O$) 0.036g L^{-1}; Ferric ammonium citrate 0.006g L^{-1}; Citric acid 0.006g L^{-1}; EDTA (di sodium Mg salt) 0.001g L^{-1}; Sodium carbonate 0.02g L^{-1}; 1ml trace metal mix.

Trace metal mix: 1ml Trace metal mix contains following constituents (in g L^{-1}): H_3BO_3 2.86 g L^{-1}; $MnCl_2.4H_2O$ 1.81 g L^{-1}; $ZnSO_4.7H_2O$ 0.222g L^{-1}; $Na_2MoO_4.2H_2O$ 0.39g L^{-1}; $CuSO_4.5H_2O$ 0.079g L^{-1}; Co $(NO_3)_2$. $6H_2O$ 0.0494 g L^{-1})

* Nitrogen source (Sodium nitrate) was not added in BG-11 media to grow nitrogen fixing cyanobacteria.

Fogg's media

Fogg's media was prepared as per the composition given by Fogg (1949).

Composition

Sodium nitrite ($NaNO_3$) 1.5g L^{-1}*; Potassium di hydrogen phosphate (KH_2PO_4) 0.2g L^{-1}; Magnesium Sulphate ($MgSO_4.7H_2O$) 0.2g L^{-1}; Calcium Chloride ($CaCl_2.2H_2O$) 0.1g L^{-1}; A5 micronutrient solution 1.0 ml, Fe -EDTA stoke solution 1.0 ml

Preparation of Fe-EDTA

Took 26.1g of EDTA (Ethylene di-amine tetra acetic acid) in 268 ml of 1N KOH solution and added to that 24.9g of $FeSO_4.7H_2O$; dissolved the contents by stirring. Aerated the solution overnight (16-18 hr). Store the Fe-EDTA (dark straw coloured) in amber coloured reagent bottle (Jacobson, 1951).

A5 micronutrient solution: Boric acid (H_3BO_3) 2.86g L^{-1}; Manganese Chloride ($MnCl_2.4H_2O$) 1.81g L^{-1}; Zinc sulphate ($ZnSO_4.4H_2O$) 0.222g L^{-1}; Sodium molybdate ($Na_2MO_4.2H_2O$) 0.0177g L^{-1}; Copper sulphate 0.027g L^{-1}.

* Nitrogen source (Sodium nitrate) was not added in Fogg's media to screen nitrogen fixing cyanobacteria.

Culture conditions

Cultures were kept under stable conditions in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent tube lamps for microalgal growth.

4.2 Screening of microalgae in pond water

Microscopic observation was done for the presence of algae in wastewater and was photomicrographed using VDO mate software and CCD camera. Identification of algae was done microscopically using identification keys of Wehr and Robert (2003) and monographs on algae by Smith (1950) and Prescott (1951). The species, which formed more that 75% of the population, were grouped in the category dominant. The species that formed 40-75% of the population were ranked common and those less than 40% in the category present.

5. Isolation of algal culture

Isolation of algal cultures was carried out according to the technique (Skinner, 1932).

5.1 Skinner's Technique

Procedure

1. Isolation of algae was carried out from wastewater samples collected from facultative pond of wastewater stabilization system located at Sanghol, Distt. Fatehgarh Sahib, Punjab, India.
2. Representative 1 ml of wastewater samples was added to a test tube containing 9 ml sterile water making a dilution corresponding to 10^{-1}. Further dilutions were prepared in a similar way u p to 10^{-6}.
3. 1 ml of appropriate wastewater dilution in sterile water was mixed in test tubes containing 9 ml BG-11 media containing 1.5 % agar, melted and cooled at 42 - 45 ^{o}C to maintain liquid condition.
4. Further dilutions were prepared in a similar way by taking 1 ml of aliquot from previous test tube and mixing it into 9 ml agar containing BG-11 media. The dilutions were made quickly before media get solidified.
5. The media was allowed to solidify after dilution which immobilized algal cells.
6. Test tubes containing immobilized algae were incubated away from direct air where they get a few hour of direct sunlight.
7. Green colonies appeared in solid media after an incubation of week after which tubes were broken carefully and agar cylinder were placed on sterile petri plates.
8. These Agar cylinders were cut and green colonies were transferred to other test tubes containing 5 ml of liquid BG-11 medium aseptically. The tubes were incubated in growth room at 28 ± 2 ^{o}C and 3000-3500 lux light intensity provided by cool white fluorescent lamps.
9. After the growth in the tubes, algal cultures were examined under microscope for identification.

5.2 Serial plate method

Isolation of algal cultures was carried out according to the serial plate method (Stanier *et al*., 1971)

Procedure

1. 10 ml suspension of algal culture was centrifuged, washed twice with 0.85% saline water and suspended to 1 ml sterile waste from which 100 µl suspension was transferred to 900 µl sterile distilled water making a dilution corresponding to 10^{-1}.
2. Further dilutions were prepared in a similar way up to 10^{-6}.
3. 100 µl of appropriate dilutions was added to the petri plate containing solidified BG-11 media and spread with the help of a sterilized spreader.
4. The plates were incubated in the growth room at 28 ± 2 °C and 3000-3500 lux light intensity provided by cool white fluorescent lamps till green colored colonies appeared on the plates, which were transferred into test tubes containing 10 ml liquid BG-11 media.
5. Axenicity of pure *Chlorella* sp. (R1) culture was regularly checked by inoculating the culture in BG-11 medium with 0.1 % yeast extract and 0.1 % glucose.

6. Development of algal consortium

Procedure

1. 4.0 ml of wastewater sample were transferred into Erlenmeyer flasks (250 ml) containing 100 ml liquid BG-11 medium.
2. The flasks were incubated in the growth room at 28 ± 2 ºC with a light-to dark cycle of 12 hours and 3000-3500 lux, light intensity provided by cool white daylight fluorescent lamps.
3. After a week of growth, identification of dominant algal types was done following monographs on algae (Smith, 1950; Prescott, 1951; Wehr and Robert, 2003).

7. Determination of Pb^{2+}, Zn^{2+} and total Cr

Concentration of Pb^{2+}, Zn^{2+} and total Cr in the sample were determined by using Atomic Absorption Spectrophotometer (GBC 932 instrument; GBC Scientific Equipment Pvt. Ltd. Dandenong, Australia). First of all samples were appropriately diluted and acidified with 1 to 2 drops of Concentrated HCl (wastewater samples were filtered with whatman filter

paper No. 42). The solid reference material was procured from Aldrich chemical company Inc. USA and the preparation of these stock solutions (1000 mg L^{-1}) was done as per *GBC* catalogue provided.

Element	Sensitivity (mg L^{-1})
Pb^{2+}	0.06
Zn^{2+}	0.008
Cr(total)	0.05

8. Growth of microalgae in medium containing metals

Metal Stocks

1. Zinc stock solution (1 g L^{-1}): 4.39 g of zinc sulphate heptahydrate was dissolved it to 1 L miliQ water and measured for actual Zn^{2+} concentration using AAS, which was further used for calculating working Zn^{2+} concentration.
2. Lead stock solution (1g L^{-1}): 1.59 g lead nitrate of analytical grade was dissolved in 1L miliQ water and measured for actual Pb^{2+} concentration using AAS, which was further used for calculating working Pb^{2+} concentration.
3. Chromium stock solution (1g L^{-1}): 2.82 g potassium dichromate of analytical grade was dissolved in 1L miliQ water and measured for actual total Cr concentration using AAS, which was further used for calculating working total Cr concentration.

8.1 Metal removal and uptake

8.1.1 Single metal conditions

Procedure

1. Three metals were selected i.e. Pb^{2+}, Zn^{2+} and total Cr, and analyzed separately for metal removal by live algae under single metal conditions
2. 100 ml of BG-11media was transferred to in 250 ml Erlenmeyer flasks. The media used was without EDTA (Ethedium diamine tetra acetic acid) which act as metal chelating agent.

3. Initial metal concentration of 1 mg L^{-1} was set in 100 ml media containing flask using 1g L^{-1} metal stock solution and subsequently increased to 5, 10, 20 and 50 mg L^{-1} in other flasks.
4. 10 ml aliquot from each flask was taken in test tubes and analyzed for initial metal concentration (C_i) by atomic absorption spectrophotometer.
5. The flasks were inoculated with 1 ml of log phase algal culture and incubated at stationary conditions in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent tube lamps.
6. At end-point of experiment after 12 days, cultures were filtered with whatman filter paper No. 42 and acidified by adding 1-2 drops of concentrated HCl
7. Final metal concentration (C_f) in the filtrate was analysed using atomic absorption spectrophotometer (*GBC 932 AA; GBC Scientific Equipment Pvt. Ltd*., *Australia*).

Calculations

The metal uptake (q) (Volesky, 1992) by the algae and bioremoval efficiency (R) (Zhang *et a*., 1998) of the algae were calculated by the following formulae.

$$q = \frac{(C_i - C_f)V}{M}$$

$$R(\%) = \frac{(C_i - C_f)}{C_i} \text{ x } 100$$

Where

q = Metal uptake (mg g^{-1}),

M = dry mass of algae (g),

R = Bioremoval efficiency (%),

C_i = Initial Conc. of metal in aqueous solution (mg L^{-1}),

C_f = final Conc. of metal in aqueous solution (mg L^{-1}),

V = Volume of culture media (L).

8.1.2 Bimetallic conditions

Procedure

1. Three metals (Pb^{2+}, Zn^{2+} and total Cr) in different combinations of Pb^{2+}-Zn^{2+}, Pb^{2+}-Cr (total) and Zn^{2+}- Cr (total) were taken for metal removal and uptake by microalgae.

2. 100 ml of BG-11media was transferred to in 250 ml Erlenmeyer flasks. The media used was without EDTA (Ethedium diamine tetra acetic acid) which act as metal chelating agent.
3. Initial metal concentration of 1 mg L^{-1} was set for first metal in 100 ml media containing flask using 1g L^{-1} metal stock solution and subsequently increased to 5, 10, 20 and 50 mg L^{-1} in other flasks keeping second metal concentration fixed in all flasks at 1 mg L^{-1}.
4. Similarly in the second set, where first heavy metal again varied with concentration from 1-50 mg L^{-1} and second metal fixed at 5 mg L^{-1}, this increase in concentration of second metal continued in each set subsequently to 10, 20 and 50 mg L^{-1} making all the possible combinations of two metals till both metals have 50 mg L^{-1} (maximum concentration range was contended to 20 mg L^{-1} for Pb^{2+} and total Cr due to precipitation of Pb and extreme algal toxicity of Cr > 20 mg L^{-1}).
5. 10 ml aliquot from each flask was taken in test tubes and analyzed for initial metal concentrations of metal 1 and metal 2 ($C1_i$ and $C2_i$) by atomic absorption spectrophotometer.
6. The flasks were inoculated with 1 ml of log phase algal culture and incubated at stationary conditions in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent tube lamps.
7. At end-point of experiment after 12 days, cultures were filtered with previously weighed whatman filter paper No. 42 (dried overnight at 100°C overnight after soaking with water to attain constant weight) and acidified by adding 1-2 drops of concentrated HCl.
8. Final metal concentration of metal 1 and metal 2 ($C1_f$ and $C2_f$) in the filtrate was analysed using atomic absorption spectrophotometer (*GBC 932 AA; GBC Scientific Equipment Pvt. Ltd., Australia*).
9. The whatman paper with algal biomass was dried at 100 °C for overnight and weighed for final weight which was subtracted from initial weight of whatman paper to find algal biomass.

Calculation

The metal uptake (q) (Volesky, 1992) by the algae and bioremoval efficiency (R) (Zhang *et a*., 1998) of the algae were calculated by the following formulae.

$$q_1 = \frac{(C1_i - C1_f)V}{M} \quad , \quad q_2 = \frac{(C2_i - C2_f)V}{M}$$

$$q_{(Total)} = q_1 + q_2$$

$$R_1(\%) = \frac{(C1_i - C1_f)}{C1_i} \times 100 \quad , \; R_2(\%) = \frac{(C2_i - C2_f)}{C2_i} \times 100$$

Where

$q_{(Total)}$ = Total metal uptake capacity for first and second metal,

q_1 and q_2 = Metal uptake of first and second metal respectively (mg g^{-1}),

M = dry biomass of algae (g),

R_1 and R_2 = Bioremoval efficiency for first and second metal respectively (%),

$C1_i$ and $C2_i$ = Initial Concentration of first and second metal respectively (mg L^{-1}),

C_f = final Concentration of first and second metal respectively (mg L^{-1}),

V = Volume of culture media (L).

8.2 Growth

8.2.1 Chlorophyll Estimation

Chlorophyll estimation of algal cultures was carried out according to the method given by Mckiney (1941).

Procedure

1. 10 ml of algal suspension was filtered through whatman filter paper No. 42 and washed with sterile double distilled water.
2. Algal biomass along with filter paper was transferred into 50 ml oak ridge centrifuge tubes (Tarsons Products Pvt. Ltd, Kolkata) and added 10 ml of 96 % methanol and the level of methanol were marked on the oak ridge centrifuge tubes.
3. The oak ridge centrifuge tubes were tightly caped, vigorously shaken and kept in water bath at 60^0 C for 30 min, which led to extraction of chlorophyll into the solution.

4. Samples were removed from the water bath and allowed to cool at room temp, made the volume again to 10 ml by adding 96% methanol and centrifuged at 8000 rpm for 10 minute.
5. Pigment of solution was analysed using spectrophotometer by comparing a sample of unknown transmission against a blank (96% methanol alone) of 100% transmission at 650 and 665nm.

Calculation

$$\text{Total Chlorophyll} = 2.55\text{x}10^{-2}.E_{650} + 0.4\text{x}10^{-2}.E_{665} \text{ mg ml}^{-1}$$

Where,

E_{650} =Absorbance at 650 nm wavelength

E_{665} = Absorbance at 665 nm wavelength

8.2.2 Unicellular algal cell count

Unicellular algal cell count was carried out according to the method given by Kaushik (1987).

Reagent

1. Lugol's reagent: In a fume hood, 10 g of KI and 5 g of I_2 were dissolved in approximately 80 mL of reagent water in a 100 ml volumetric flask, mixed until the chemicals are completely dissolved followed by addition of 10 mL of glacial acetic acid. Final volume was made to 100 ml with double distilled water.

Procedure

1. 5 ml of algal culture (unicellular) was centrifuged and pellet was washed with double distilled water and re-suspended into 5 ml sterile distilled water.
2. The cell suspension was appropriately diluted and stained with Lugol's reagent, which make cells darker and heavier to settle.
3. One drop of cell suspension was put on each side of the central groove on haemocytometer with 1 ml pipette and special cover glass was placed and pressed it uniformly then observed under microscope at 40x.
4. The haemocytometer was having $1mm^2$ area and 0.1mm depth where central $1mm^2$ area was divided in 25 squares each having 0.04 mm^2 area, which were further subdivided into 16 squares, each of 0.0025 mm^2 size.

5. Average No. of cells in a square having an area of 0.04 mm^2 were counted.

Calculation

No. of cells ml^{-1} of sample = $n/4 \times 10^6$

Where,

n = average No. of cells/ 0.04 mm^2

9. Microcosms experiment setup

1. 15 litres wastewater sample collected from Wastewater stabilization ponds at village Sanghol, Distt. Fatehgarh, Punjab, India. was brought to the laboratory and diluted with BG-11 (half strength) media in the ratio 1:3.
2. Eight litres of diluted wastewater was suspended separately into eight plastic tubs of 10 L capacity divided into two sets of two tubs each.
3. Tub 1 of each set was kept as control where Tub 2 was inoculated with 50 ml log phase culture of *Chlorella* sp. (R1).
4. All the tubs were covered with transparent pierced plastic sheet from which air and light can pass.
5. One set of two tubs containing control tub (Tub 1) and tub inoculated with *Chlorella* sp. (R1) (Tub2) was placed in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent tube lamps while the other set was kept in natural outdoor conditions of light and atmosphere.
6. Representative samples from each tub were drown at start of the experiment and an interval of ten days and characterized for temperature, pH, Electrical conductivity, Salinity, Biochemical oxygen demand, Chemical oxygen demand.
7. Loss of water by evaporation was maintained by addition of sterile distilled water.

10. Metal removal by silica immobilized alga

10.1 Immobilization of alga on silica

Procedure

1. Algal culture to be immobilized was grown in BG-11 media for 12 days.
2. Algal biomass was harvested by centrifugation and washed twice with sterile double distilled water and finally with 0.12N HCl to remove all nutrients from the cell wall.

3. The algal cells were collected in 100 ml beaker and dried in an oven at 100 °C overnight.
4. The dried biomass was transferred to pestle and mortar and ground to powder.
5. 160 mg of dried algal powder was added to 2 g Silica gel Devisil, 30-60 mesh (Sigma-Aldrich, Mumbai) and wetted with minimal amount of water to make a paste which was then heated at 105 ^{o}C for 20 minute to drive off the water.
6. Above wetting, mixing and drying steps were repeated three to four times for better contact between algae and silica surface.
7. The dried silica-alga particles were used to pack the columns for metal removal studies.
8. Same procedure was used for immobilization of microalgal consortium (CP1) whereas, silica gel G, 350 mesh (S. D. Fine Chemicals) was used.

10.2 Removal of metals (Pb^{2+}, Zn^{2+} and total Cr) from synthetic solution by alga immobilized on silica

Reagents

1. Hydrochloric acid (0.12 N): 5 ml of concentrated HCl was added to double distilled water and volume made up to 500 ml.

Metal stocks

2. Zinc stock solution (1 g L^{-1}): 4.39 g of zinc sulphate heptahydrate was dissolved it to 1 L miliQ water and measured for actual Zn^{2+} concentration using AAS, which was further used for calculating working Zn^{2+} concentration.
3. Lead stock solution (1g L^{-1}): 1.59 g lead nitrate of analytical grade was dissolved in 1L miliQ water and measured for actual Pb^{2+} concentration using AAS, which was further used for calculating working Pb^{2+} concentration.
4. Chromium stock solution (1g L^{-1}): 2.82 g potassium dichromate of analytical grade was dissolved in 1L miliQ water and measured for actual total Cr concentration using AAS, which was further used for calculating working total Cr concentration.

Procedure

1. 2 g of dried algal biomass immobilized on silica was packed in plastic column (Diameter = 1cm, Height = 5 cm) after putting glass wool at the mouth of column as a support to biomass.

2. At start of the experiment 50 ml of miliQ water was passed through the column followed by another 50 ml of 0.12 N HCl to activate the column.
3. About 100 ml of Pb solution (156.6 mg L^{-1}) was gravity fed to the column using autopipette and tips which were collected in 5 ml fractions after passing through the column.
4. Fractions collected from outlet stream in test tubes were analyzed for metal concentration in solution using AAS.
5. The time interval for passing 5 ml metal solution was reported every time to calculate average flow rate of solution.
6. As the outlet stream solution attained same metal concentration of inlet solution, no further metal solution was passed from the column.
7. The adsorbed Pb was recovered in 0.12 N HCl and analysed for metal concentration.
8. The steps 2-7 were repeated with same Pb solution (156.6 mg L^{-1}) to analyze column performance for two more cycles.
9. The above experiment was also performed similarly with synthetic Zn^{2+} (82.6 mg L^{-1}) and total Cr (92.6 mg L^{-1}) solution.
10. The effect of increase in flow rate was tested during this study using columns with reduced bed height by half after packing 1 gm of biomass in same plastic column with identical dimensions. The experiment was performed with synthetic Pb^{2+} (95.6 mg L-1), Zn^{2+} (70.0 mg L^{-1}) and total Cr (75.6 mg L^{-1}).
11. The study was carried out following same procedure to determine removal of total chromium from chrome electroplating industrial wastewater by silica immobilized *Chlorella* sp. (R1) in packed bed column. Half strength electroplating industrial wastewater (Gudzey pvt. Ltd, Patial, India) containing total Cr of 341 mg L^{-1} was passed through the column containing 2 g immobilized *Chlorella* sp. (R1) biomass.
12. The study was carried out following same procedure to determine removal of Pb^{2+} from aqueous solution by silica immobilised microalgal consortium (CP1) in packed bed column. Synthetic lead solution containing Pb^{2+} of 33.90 mg L^{-1} was passed through the column containing 1.5 g silica ( ) immobilized microalgal consortium (CP1) biomass.

11. Statistical data analysis

The various statistical parameters were analysed (Rao, 1996) and using Mini Tab Software and Microsoft Excel.

Variance

The variance is measured as the square of the units in which the variable X is measured. For example, if X is the height in centimeters (cm), the variance will be measured in cm2 (square centimeters). The formula for variance is:

$$Variance = \frac{\sum (Xi - \overline{X})^2}{n} = \frac{\sum Xi^2 - \overline{nX}^2}{n}$$

Sum of the squares of the deviations of individual values from the mean x ¸ sample size where n is the number of observations; x is the arithmetic mean of the observations of X2s are the individual observations – x1,x2…….xi……xn.

Standard Deviation

It is convenient to have a measure of variation expressed in the original units of X and this can be done by taking the square root of the variance. This quantity is known as the standard deviation and is, $SD = \sqrt{Variance}$

Standard Error

The standard error (SE) is a measure of the variation or dispersion of the means of a set of measurements. It is, therefore, smaller than the standard deviation of a single series of measurements form the same of population. It is used to compare means with one another.

$$\text{Formula for S2} \qquad = Variance = \frac{\sum (Xi - \overline{X})^2}{n-1}$$

$$= (\sum Xi^2 - \overline{nX}^2 / (n-1) = (SS - CF) \div (n-1)$$

Standard deviation = square root of variance = $\sqrt{S^2}$

Standard error = $\sqrt{Variance / sample\ size}$ = $\sqrt{(S^2 / n)}$

Standard error is the standard deviation of the means of measurements. It is an indication of the magnitude of variation between sample mean values. Standard error is also called the standard deviation of the mean

Probit analysis (Finney, 1952)

Probit analysis is a type of regression used with binomial response variables. It is very similar to logit, but is preferred when data are normally distributed. The most common outcome of a dose-response experiment using probit analysis is 50% lethal concentration (LC_{50}).

Procedure using hand calculations

1. Probits were determined by looking up those corresponding to the % responded in Fanny's table (Finney, 1952)
2. Log_{10} was taken of the concentrations.
3. Graph was drown for probits versus log_{10} values of the concentrations and regression line was fitted.
4. Probit was found of 5 in the y-axis, then move down to the x-axis to find the log_{10} of the concentration associated with it.
5. Took the Inverse of the log_{10} of the concentration to get LC_{50}

RESULTS

1. Monitoring of microalgal diversity of wastewater

The role of microalgae as a dominating component of wastewater treatment in facultative pond was monitored on a monthly basis from wastewater stabilization ponds (WSP). Onsite case study was conducted on pond system at village Sanghol, Distt. Fatehgarh Sahib, Punjab, India, to study its microalgal diversity and wastewater treatment potential. The village Sanghol also known as Ucha Pind Sanghol with an approximate population 53,397 is located at a latitude of 30.7833, longitude 76.3833 and altitude (Feet) 843 which lie in time zone (est) of UTC+5:30. The day temperature ranges from 45°C in May -June to 4°C in December-January and has a sub-tropical continental monsoon climate with satisfactory rain fall.

1.1Microalgal diversity

The facultative and maturation ponds of wastewater stabilization pond system maintained its green color throughout the year. The diversity of microalgae was studied in wastewater samples microscopically over complete observation period and a total of microalgal genera were identified belonging to different typological groups Cyanophyta, Chlorophyta and Bacillariophyta. Chlorophyta were the dominant group in facultative ponds and representative members were identified as *Chlorella* sp., *Chlorococcum* sp., *Closteriopsis* sp. and *Chlamydomonas* sp. (Table 4, 5).

The genera *Chlorella* sp. and *Chlamydomonas* sp. were the dominant forms with maximum cell count of *Chlorella* sp. was observed in the month of May to September (Table 4, 5). The other group of microalgae which equally dominated the facultative pond system was Cyanophyta which was represented by *Nostoc* sp. *Lyngbya* sp. *Gloeocapsa* sp. *and Myxosarcina* sp. (Table 4, 5) which dominated in the pond system from February to May whereas, *Nostoc* sp. was observed throughout the observation period. *Lyngbya* sp. was reported from microalgal blooms formed in the pond system in winters. The genera belonging to class Bacillariophyta *(Diatoms)* were reported only in the month of December whereas, it was totally absent during the months from January to May and then from August to November (Table 5). Relative dominance of microalgal species revealed that *Chlorella* sp. was present during the whole sampling period as one of the most dominating species followed by *Chlamydomonas*, *Lyngbya* and *Diatoms*.

Table 4. Identification of microalgae found in wastewater sample collected from Wastewater stabilization pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab.

Phylum or Division/Class	Genus	Identification Keys (Smith, 1950; Prescott, 1951; Wehr and Robert, 2003)
Cyanophyta /Cyanophyceae	*Nostoc* sp.	Filaments of spherical or barrel-shaped cells, The bent, kinked, or coiled filaments are long, isopolar, and held together by firm mucilage, heterocysts are solitary, barrel-shaped or spherical, and may be intercalary or located at the ends of the trichomes.
	Lyngbya sp.	Filamentous, composed of a single series of cells, filamentous usually wider than 6 µm surrounded by a tough covering or sheath
Chlorophyta /Chlorophyceae	*Chlorella* sp.	Unicellular green microalgae with simple structure containing a relatively large cup shaped chloroplast and a small terminal nucleolus
	Chlorococcum sp.	Unicellular with spherical or slightly oblong cells of varied size, solitary or in irregular shape, mucilage is thin and inconspicuous. Each cell has a single cup-shaped, parietal chloroplast with a single pyrenoid.
	Closteriopsis sp.	Unicellular green microalgae, needle like, acute apices with no mucilage, single chloroplast
	Chlamydomonas sp.	Unicellular flagellates, 10 micrometer in diameter that swims with two flagella, cup-shaped chloroplast, a large pyrenoid, and an "eyespot
Bacillariophyta / Diatomophyceae	*All organisms are in the phylum Bacillariophyta and their abundance was not consistent and not significant*	Unicellular, encased within a unique cell wall made of silica (hydrated silicon dioxide) called a frustula. wide diversity in form, some quite beautiful and ornate, but usually consist of two asymmetrical sides.

Two unialgal cultures (R1, R2) and five consortia of mixed microalgal culture (R3, R5, R6, R9 and CP1) were developed by isolating microalgae from wastewater samples collected from microalgal stabilization pond using classical stab technique and serial plating on solid BG-11 media plates.

Microalgal cultures R1 and R2 were identified as pure cultures of *Chlorella* sp. (Plate 1, 2). and *Chlorococcum* sp. respectively where as, microalgal culture R3 was found to be a mixture of *Chlorella* and *Chlorococcum* (Plate 1). The other microalgal cultures R5 was found to contain *Nostoc* sp. in association with *Chlorella* sp. whereas R6 contained mixture of three cyanobacteria (*Myxosarcina* sp., *Gloeocapsa* sp. *and Chlamydomonas*

sp.) and R9 contained only *Myxosarcina* sp., *Gloeocapsa* sp. (Table 8). Consortium (CP1) consisted of mix microalgal composition dominated by *Chlorella* > *Chlamydomonas* > *Lyngbya* sp. (Table 8).

Table 5. Seasonal variation in microalgal community of wastewater sample collected from Wastewater stabilization Pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab.

Algal Species	**Month**									
	A	S	O	N	D	J	F	M	A	M
***Nostoc* sp.**	+	+	+	+	+	+	+	+	+	+
***Chlorella* sp.**	+++	+++	+	+	+	+	+	++	++	+++
***Chlorococcum* sp.**	++	++	-	-	-	-	-	-	++	++
***Lyngbya* sp.**	-	+	++	+	+	+	-	-	-	-
***Chlamydomonas* sp.**	+	+	+	+	+	+	+	+	++	++
***Clostropsis* sp.**	+	+	-	-	-	+	-	-	-	-
Diatoms	-	-	-	-	+	-	-	-	-	-

+++ = Dominant; ++ = Common; + = Present; - = Absent.

1.2 Characterization of wastewater

1.1.1 Wastewater stabilization pond (WSP)

Onsite case study for ten months from August 2004 to May 2005 was carried out to monitor the potential of microalgal based wastewater stabilization ponds in treatment of domestic wastewater generated from the village of Sanghol Distt. Fatehgarh sahib, Punjab, India. The system constituted an anaerobic pond (Length = 6.55 m x Breadth = 3.54 m), a facultative pond (Length = 84m x Breadth = 53m x Depth = 1.22 m) and a maturation pond (Length = 84m x Breadth = 50m x Depth = 1.83 m) in series. The wastewater generated was applied directly to the anaerobic pond where the settling of sludge and anaerobic treatment takes place with the help of anaerobic bacteria. Anaerobically treated water was passed to Pond 1 which is a facultative pond where the microalgae and duckweeds utilise inorganic nutrient for their growth and produce nascent oxygen during photosynthesis and thus augments complete oxidation of organic compounds lowering COD and BOD_5. The primary focus under the presented study was given to the role of facultative ponds which receives COD and BOD_5 load from anaerobic ponds and carried out treatment using microalgae and phytoplankton for the end use. After a residence time of 12-14 days the treated wastewater was passed to pond 2 which acted as a maturation pond in wastewater treatment system for final treatment of treated water.

1.1.2 Monitoring of wastewater characteristics

Facultative pond inlet and outlet wastewater samples were periodically characterized at monthly interval for physicochemical parameters (temperature, pH, conductivity, salinity, dissolved oxygen, chemical oxygen demand and biochemical oxygen demand) alongwith enumeration of culturable bacteria and metals (Total Cr, Pb^{2+} and Zn^{2+}) to monitor wastewater treatment potential of pond system (Table 6).

1.1.3 Temperature

The midday water temperature varied considerably with respect to seasonal variations (Table 6). The inlet wastewater temperature varied from a minimum of 12.7°C in the month of January to a maximum of 32°C in August with an average water temperature of 23.7°C during complete observation period, whereas pond outlet water temperature ranged from a minimum of 12°C to 33.6°C in the month of January and August respectively (Table 6). It has also been observed that both inlet and outlet stream had same temperature, with the temperature of outlet water was slightly higher (0.2 - 1°C) due to microalgal growth and microbial metabolic activities.

1.1.4 pH

The pH of wastewater was alkaline throughout however it tends to become more alkaline after treatment (Table 6). The pH of inlet wastewater varied from 7.83 to 8.73 with an average of 8.34 over complete observation period whereas, a significant increase in pH was observed after treatment which was in the range of 8.52 to 10.72 with an average of 9.56 showing an increase of more than one unit (Table 6, 7).

1.1.5 Conductivity and salinity

Since conductivity and salinity are important parameters to be considered for water used for irrigation, the conductivity and salinity of facultative pond inlet and outlet wastewater varied considerably during complete observation period. Conductivity of inlet wastewater ranged from 1.9 to 4 mS cm^{-1} and 1.7 to 2 mS cm^{-1} for outlet wastewater with an average of 3.63 and 1.92 mS cm^{-1} for inlet and outlet respectively. Conductivity decreased after treatment and varied from minimum reduction of 1.4 mS cm^{-1} to maximum 2.78 in the month of October and March respectively. Similarly reduction in salinity from 1 - 2.3‰ was also reported in treated wastewater over the entire observation period. An average

47% and 48% reduction in conductivity and salinity was observed after treatment respectively (Table 7).

1.1.6 Dissolved oxygen

The inlet wastewater exhibited oxygen limiting conditions with dissolved oxygen (D.O.) ranging from 0.55 to 4.04 mg L^{-1} with an average of 1.98 mg L^{-1} during complete observation period. However due to photosynthetic fixation of oxygen microalgae present in the facultative pond, the treated wastewater showed significant addition of dissolved oxygen. The D.O. of pond outlet water increased significantly and varied widely from 9 to 14 mg L^{-1} during the study period indicating complete saturation of water or even excess of oxygen trapped in the form of bubbles (Table 6, 7). This marked increase in dissolved oxygen clearly showed the role of microalgae in converting oxygen limiting conditions of inlet wastewater to oxygen rich outlet water.

1.1.7 Chemical oxygen demand

The Chemical oxygen demand (COD) which is used as an indicator of pollution in the water had marked variation during complete study period and its removal in outlet water samples in comparison to inlet samples showed pond treatment potential. The COD values were higher in influent wastewater which varied from 183.2-451.2 mg L^{-1} with an average of 303.5 mg L^{-1}, which was reduced to a range of 80.0-281.6 mg L^{-1} with an average of 134.6 mg L^{-1}, in outlet water. The facultative pond reduced wastewater COD with minimum 41% to maximum 93% with an average removal of 50.26% as observed during complete study period (Table 6, 7). During monsoon month of September, COD of treated wastewater was high with an average value of 280 mg L^{-1} which was high mainly due to increase in the drainage of dissolved rural waste (Fig. 4).

1.1.8 Biochemical oxygen demand

The five day Biochemical oxygen demand (BOD_5) was used as an indicator of organic pollution in wastewater and showed marked variation in pond inlet and outlet wastewater during complete study period. The BOD_5 variation in inlet wastewater was observed in the range of 48.0 mg L^{-1} to 365.8 mg L^{-1} with an average of 185.4 mg L^{-1} whereas, the treated wastewater which was finally released from facultative pond had a minimum BOD_5 of 20 mg L^{-1} in the month of May and a maximum of 114.2 mg L^{-1} in month of September (Fig. 5).

Table 6. Characterization of wastewater sample collected from Wastewater stabilization pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab, during August 2004 to May 2005.

Parameter analysed		**Months**									
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Temp (C°)	Inlet	32.0	32.0	27.1	17.70	17.5	12.7	22.5	24.2	24	27.8
	Outlet	33.6	32.7	27.5	19.2	19.2	12.0	22.9	24.5	23.4	28.0
pH	Inlet	7.83	7.98	8.49	8.11	8.17	8.23	8.57	8.62	8.30	8.73
	Outlet	9.30	9.23	8.52	9.12	10.72	9.22	9.26	9.36	8.90	8.26
Cond (mS)	Inlet	3.50	3.57	3.52	3.53	4.00	4.49	1.94	4.67	3.61	3.47
	Outlet	2.04	2.02	2.12	1.78	1.88	1.97	1.75	1.89	1.85	1.96
Salinity (‰)	Inlet	1.8	1.9	1.8	1.8	2.1	2.3	1.0	2.5	1.9	1.7
	Outlet	1.0	1.0	1.1	0.9	1.0	1.0	0.9	1.0	0.9	0.9
D.O ($mg\ L^{-1}$)	Inlet	0.55	0.58	0.58	2.23	3.47	4.04	3.24	1.20	-	-
	Outlet	14	14	11.35	10.08	11.60	12.00	11.50	9.80	-	-
COD ($mg\ L^{-1}$)	Inlet	304	230.4	451.2	-	183.2	278.4	272	373.3	-	336.0
	Outlet	80.0	281.6	220.8	-	12.8	51.2	128	213.3	-	89.6
BOD ($mg\ L^{-1}$)	Inlet	149.2	134.2	89.80	165.4	152.1	365.8	-	-	379.1	48
	Outlet	34.7	114.2	32.2	46.7	70.1	76.3	-	-	64.1	20
Culturable Bacterial count x 10^3 ($cfu\ ml^{-1}$)	Inlet	76.2	76.5	31.5	85.5	65	370	110	87	53	76
	Outlet	1.0	1.8	5.0	5.0	31.6	73	86	11	8	9
Total Cr ($mg\ L^{-1}$)	Inlet	-	0.032	<0.02	0.071	0.069	0.051	0.028	<0.02	<0.02	<0.02
	Outlet	-	<0.02	0.096	<0.02	0.018	0.019	<0.02	<0.02	<0.02	<0.02
Pb^{2+} ($mg\ L^{-1}$)	Inlet	-	0.106	<0.02	0.141	<0.02	<0.02	<0.02	0.18	0.04	0.054
	Outlet	-	0.134	<0.02	0.163	<0.02	0.016	<0.02	0.05	>0.02	>0.02
Zn^{2+} ($mg\ L^{-1}$)	Inlet	-	0.021	0.056	0.250	<0.02	<0.02	<0.02	0.041	0.061	0.051
	Outlet	-	<0.02	0.083	0.068	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02

Overall after a residence time of 12-14 days, the facultative pond effluent wastewater was released with an average BOD_5 of 57.3 mg L^{-1}. The reduction in BOD_5 was from 53 to 79% during the entire period of study (Table 6, 7). In the month of September BOD_5 of treated wastewater was high (114 mg L^{-1}) and could be due to increase in the drainage of dissolved rural waste (Table 6).

Table 7. Treatment potential of wastewater in Wastewater stabilization pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab.

Parameter analysed	Inlet		Outlet		Removal (%)
	Mean	Range	Mean	range	
pH	8.34	7.83-8.73	9.56	8.52-10.72	-10.67
EC (mS)	3.63	1.94-4.67	1.92	1.75-2.12	46.94
Salinity (‰)	1.88	1.00-2.50	0.97	0.90-1.10	48.40
DO (mg L^{-1})	1.98	0.55-4.04	11.79	9.80-14.00	-816
COD (mg L^{-1})	303.5	183.2-451.2	134.6	80.0-281.6	50.26
BOD_5 (mg L^{-1})	185.4	48.0-365.8	57.3	20.0-114.2	69.10
Culturable Bacterial count x 10^3 (cfu ml^{-1})	103	31.5 -370	23.1	1 -86	77.54

* Negative values indicate that concentrations in final effluent were higher than those in untreated wastewater

1.1.9 Culturable bacterial cell count

Facultative ponds are mainly responsible for reduction of pathogenic bacteria. The culturable bacterial cell count in pond inlet wastewater ranged from 31.5 x 10^3 to 370 x 10^3 cfu ml^{-1} during complete observation period with an average of 103 x 10^3 cfu ml^{-1} which was drastically reduced in outlet wastewater to a minimum of 1x 10^3 cfu ml^{-1} to a maximum of 86 x 10^3 cfu ml^{-1} (Table 6, 7). The inlet wastewater in January and February contained highest bacterial load of 370 x10^3 and 110 x 10^3 and also released wastewater with high bacterial count of 73 x 10^3 and 83 x 10^3 cfu ml^{-1} respectively (Table 6, 7).

1.1.10 Removal of heavy metals (Total Cr, Pb^{2+} and Zn^{2+})

The facultative pond was also reported to remove heavy metals from wastewater and the performance is dependent on the biotic community present in the treatment pond, which is exposed to different pollutants. Concentration of heavy metals such as Total Cr, Pb^{2+} and Zn^{2+} were with in the range of permissible limit fixed by Punjab Pollution Control Board (PPCB), which were < 0.1, < 0.1 and < 0.5 mg L^{-1} for total Cr, Pb^{2+} and Zn^{2+} respectively, Reduction in heavy metal concentration was reported in outlet water in comparison to inlet after treatment in facultative pond (Table 6). In the microalgal stabilization ponds there was 73% removal of Pb^{2+} and 72% removal of Zn^{2+} especially in the months of November and March respectively, whereas 28 to 72% removal of total Cr removal was observed (Table 6).

1.1.11 Wastewater treatment potential of WSP

Wastewater stabilization pond showed effective treatment potential in treating domestic wastewater as observed following the water characteristics of inlet and outlet wastewater (Table 7). It has been observed that outlet water temperature was slightly higher than inlet by 0.2 - 1°C, indicating microbial metabolic activities whereas pH was increased after treatment by one unit from an average of 9.56 (Table 6, 7). The facultative pond reduced the level of soluble salts as indicated by corresponding reduction in wastewater conductivity and salinity where conductivity was reduced in outlet wastewater from minimum 1.4 mS cm^{-1} to maximum 2.78 in the month of October and March respectively whereas 1 - 2.3‰ reduction in salinity was also reported after treatment (Table 7). The dissolved oxygen of outlet wastewater increased significantly and varied widely from 9 to 14 mg L^{-1} during the study period indicating complete saturation of water or even excess of oxygen trapped in the form of bubbles (Table 6, 7). This marked increase in dissolved oxygen clearly showed the role of microalgae in converting oxygen limiting conditions of inlet wastewater to oxygen rich outlet water.

The treatment of domestic wastewater in terms of removal of organic and inorganic pollutants as indicated by COD and BOD_5 of wastewater also showed significant reduction. The facultative pond reduced wastewater COD with minimum 41% to maximum 93% with an average removal of 50.26% as observed during complete study period (Table 6, 7) whereas, this reduction in BOD_5 varied between 53 to 79% during the entire period of study with

average of 69.10% (Table 6, 7). The reduction in BOD_5 after treatment was found in correlation with the reduction in wastewater bacterial load where an average 77.54% reduction in total culturable bacterial count was observed (Table 7). The inlet wastewater in January and February contained highest bacterial load of 370 $x10^3$ and 110 x 10^3 and also released wastewater with high bacterial count of 73 x 10^3 and 83 x 10^3 cfu ml^{-1} respectively (Table 6). Concentration of heavy metals such as Total Cr, Pb^{2+} and Zn^{2+} were with in the range of permissible limit fixed by Punjab Pollution Control Board (PPCB) but still heavy metal removal was reported in outlet water by 73% of Pb^{2+} and 72% removal of Zn^{2+} especially in the months of November and March respectively, whereas 28 to 72% removal of total Cr removal was observed (Table 6).

2. Removal of metal (Zn^{2+}, Pb^{2+} and total Cr) by selected microalgae

2.1 Selection of Chlorella sp. (R1) and algal consortium (CP1) for metal removal studies

Two unialgal cultures (R1, R2) and four mixed microalgal cultures (R3, R5, R6, R9) and microalgal consortium (CP1) developed from wastewater samples were screened for their ability to remove metals (Total Cr, Pb^{2+} and Zn^{2+}) during growth. The unialgal cultures R1 and R2 were identified as pure cultures of *Chlorella* sp. and *Chlorococcum* sp. respectively whereas, mixed microalgal culture R3 containing *Chlorella* sp. and *Chlorococcum* sp., R5 (*Nostoc* sp. and *Chlorella* sp.), R6 (*Myxosarcina* sp., *Gloeocapsa* sp. and *Chlamydomonas* sp.), R9 (*Myxosarcina* sp., *Gloeocapsa* sp.) and microalgal consortium (CP1) (*Chlorella* sp. > *Chlamydomonas* > *Lyngbya* sp.). Microalgal cultures were grown in 100 ml BG-11 (without EDTA) medium containing single metal at fixed initial concentration of 5 mg L^{-1}. The study was carried out to select potential microalgal candidate for metal removal under pond conditions.

Microalgal cultures developed from pond wastewater exhibited varying ability for Pb^{2+} removal at a constant external metal concentration of 5 mg L^{-1}. The microalgal culture R1 (*Chlorella* sp.), R2 (*Chlorococcum* sp.) and R5 (*Nostoc* sp., *Chlorella* sp.) removed greater than 62% of Pb^{2+}, where 68.5% removal as observed by R5 (Fig. 6). The consortium (CP1) has least ability for lead removal whereas, microalgal culture R1 which is a pure culture of *Chlorella* sp. was found as a good candidate for Pb^{2+} removal (Fig. 6). The *Chlorella* sp. in culture (R1) not only sowed good Pb^{2+} removal ability but also removed

appreciable amount of Zn^{2+} by removing 67.12% of Zn^{2+} from medium (Fig. 7). All other microalgal cultures R2, R3, R5, R6 and CP1 except R9 showed more than 50% Zn^{2+} removal (Fig. 7). Cyanobacterial mixture of *Myxosarcina* and *Gloeocapsa* in culture R9 had least ability of 34.84% Zn^{2+} removal. Consortium (CP1) which represented indigenously developed microalgae was found to have poor ability for Pb^{2+} but highest (68%) for Zn^{2+} removal (Fig. 7). Among all only microalgal culture R9, which is a mixture of two cyanobacteria *Myxosarcina* sp. and *Gloeocapsa* sp., *Chlorella* sp. (R1) and consortium (CP1) showed 37.2%, 10% and 20% removal of total chromium respectively from medium containing 5 mg L^{-1} of external metal concentration after 12 days of incubation (Fig. 8).

Table 8. Development of algal cultures from wastewater sample collected from Wastewater stabilization pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab.

Algal Culture	*Microalgal Types*
CP1	*Chlorella* sp., *Chlamydomonas* sp., *Lyngbya* sp.
R1	*Chlorella* sp.
R2	*Chlorococcum* sp.
R3	*Chlorococcum* sp., *Chlorella* sp.
R5	*Chlorella* sp., *Nostoc* sp.
R6	*Myxosarcina* sp., *Gloeocapsa* sp., *Chlamydomonas* sp.
R9	*Gloeocapsa* sp. *and Myxosarcina* sp.

2.2 Metal removal potential of Chlorella sp. (R1) in single metal solution

To determine the metal removal potential of *Chlorella* sp. (R1) which dominated the stabilization pond system during 10 months period, was isolated as pure culture and studied for its Pb^{2+}, Zn^{2+} and total Cr removal over a concentrations range of 1-50 mg L^{-1} maintained in culture medium under flask conditions at an average pH (9 ± 0.2) of pond water. The pure culture of *Chlorella* sp. (R1) showed 3 days of lag phase followed by 10 days of exponential growth phase in BG-11 medium under controlled laboratory conditions (Fig. 9). Overall *Chlorella* sp. (R1) showed better removal for Zn^{2+} followed by Pb^{2+} and total Cr.

2.2.1 Effect of varying Zn^{2+} concentration on growth and its removal

The effect of varying concentration of Zn^{2+} was studied following the unicellular microalgal cell count using haemocytometer and chlorophyll content as indicator of growth. A constant decline in cell number from 538 x 10^5 to 6 x 10^5 cells ml^{-1} and chlorophyll content from

$101.61x10^{-4}$ to $9.8x\ 10^{-4}$ mg ml^{-1} by increasing Zn^{2+} concentration from 1 mg L^{-1} to 50 mg L^{-1} indicating Zn^{2+} toxicity to *Chlorella* sp. (R1) (Table 4). *Chlorella* sp. (R1) cell number was slightly increased from 432 x 10^5 to 538 x 10^5 cells ml^{-1} by increasing Zn^{2+} concentration from 1 to 5 mg L^{-1} showing an encouraging effect of Zn^{2+} on cell proliferation (Fig. 10). The cell count decreased consistently by further increasing Zn^{2+} concentration greater than 5 mg L^{-1} and thus 50% lethal concentration for Zn^{2+} was calculated to be 10.25 mg L^{-1}. Effect of 50 mg L^{-1} Zn^{2+} concentration revealed 98.6% reduction in cell count after 12 days of incubation. The chlorophyll content of microalgal cultures as measured after 12 days of incubation revealed the similar patron along with the change in Zn^{2+} concentration where an increase in chlorophyll content was observed from 101.61 x 10^{-4} to 165 x 10^{-4} mg ml^{-1} by increasing Zn^{2+} from 1 mg L^{-1} to 5 mg L^{-1} respectively (Table 9). The cultures maintained a consistent range of 165 x 10^{-4} -175 x 10^{-4} mg ml^{-1} chlorophyll at Zn^{2+} concentration range of 5-20 mg L^{-1} despite of a decrease in cell count (Table 4). The chlorophyll showed similar drastic decline as for cell count by 94% on further increasing Zn^{2+} concentration to 50 mg L^{-1}. *Chlorella* sp. (R1) showed maximum removal efficiency for Zn^{2+} with 60-70% removal of Zn^{2+} was observed from culture medium concentration from 5-20 mg L^{-1} Zn^{2+} which further declined to 42% at 50 mg L^{-1} (Fig. 10). Interestingly, the decrease of cell count was not affecting the Zn^{2+} removal potential till 20 mg L^{-1}, supporting the contribution of dead biomass in metal removal. The specific metal uptake (q) for Zn^{2+} increased concomitantly with increase in metal concentration in medium with maximum metal uptake (q_{max}) of 41.75 for Zn^{2+} as calculated by mass balance for live *Chlorella* sp. (R1) biomass (Table 9).

Table 9. Effect of Zn^{2+} concentration on metal removal and growth of *Chlorella* sp. (R1).

Parameter Analysed	Zn^{2+} (mg L^{-1})				
	1	5	10	20	50
Metal removal efficiency (%)	10.49	70.29	60.91	71.59	33.76
Metal uptake (mg g^{-1})	0.07	6.01	8.44	19.8	41.75
Chlorophyll x 10^{-4} (mg ml^{-1})	201.61± 0.00023	165 ± 0.0009	155 ± 0.0021	105 ± 0.00025	98 ± 0.000037
Microalgal cell (Count x 10^5 ml^{-1})	432	538	208	21	6

2.2.2 Effect of varying Pb^{2+} concentration on growth and its removal

The microalgal cell count and chlorophyll as measured from lead containing medium after 12 days of *Chlorella* sp. (R1) growth indicated no signs of Pb^{2+} toxicity up to 20 mg L^{-1} (Fig. 11). Pb^{2+} demonstrated positive effect of increasing Pb^{2+} concentration on *Chlorella* sp. (R1) cell number which increased from 118 x 10^5 to 278 x 10^5 cells ml^{-1} by increasing external metal concentration whereas this increase in chlorophyll was 27.46 x 10^{-4} to 52.79 x 10^{-4} mg ml^{-1} from 1 to 5 mg L^{-1} (Table 10). The increased values of *Chlorella* sp. (R1) cell count and chlorophyll was maintained with slight reduction by further increasing external metal concentration greater than 5 mg L^{-1} (Table 10). With the growth of *Chlorella* sp. (R1), Lead was maximally removed to 66% from culture medium containing 1 mg L^{-1} whereas, 45 to 50% removal efficiency was maintained for 5 to 20 mg L^{-1} of external lead concentration which directly correlated with the growth of *Chlorella* sp. (R1) and indicating the involvement of live cells in whole Pb^{2+} removal till 20 mg L^{-1} of metal concentration (Fig. 11). *Chlorella* sp. (R1) demonstrated high removal efficiency for Zn^{2+} then Pb^{2+} whereas, specific metal uptake (q) for Pb^{2+} increased concomitantly with increase in metal concentration in medium with maximum metal uptake (q_{max}) of 34.36 for Pb^{2+} as calculated by mass balance for live *Chlorella* sp. (R1) biomass (Table 10).

Table 10. Effect of Pb^{2+} concentration on metal removal and growth of *Chlorella* sp. (R1).

Parameter Analysed	Pb^{2+} (mg L^{-1})			
	1	5	10	20
Metal removal efficiency (%)	66.29	49.87	45.44	45.3
Metal uptake (mg g^{-1})	3.08	14.14	17.66	34.36
Chlorophyll x 10^{-4} (mg ml^{-1})	27.46 ± 0.0001	52.79 ± 0.0002	44.88 ± 0.0006	62.42 ± 0.0010
Microalgal cells (Count x 10^5 ml^{-1})	118	235	208	278

2.2.3 Effect of varying total Cr concentration on growth and its removal

The results demonstrated the toxic effect of increasing total chromium concentration on the growth of *Chlorella* sp. (R1) and its potential of chromium remediation from wastewater. In

batch study where *Chlorella* sp. (R1) was grown in the presence of total chromium at variable concentrations which demonstrated that addition of chromium to the medium at 1 mg L^{-1} showed increase in *Chlorella* sp. (R1) cell count to 93.6 x 10^5 cell ml^{-1} over 88 x 10^5 cell ml^{-1} in non-metallic control which however declined drastically to 5.3 x 10^5 cell ml^{-1} in medium containing 5 mg L^{-1} of total Cr (Table 11).

This cell count of 4.8 x 10^5 to 2.5 x 10^5 cells as observed from medium containing 10 and 20 mg L^{-1} demonstrated the presence of resistance cells and dead cell debris. LC_{50} of 4.34 mg L^{-1} total Cr was derived by probit analysis for *Chlorella* sp. (R1). In batch study where *Chlorella* sp. (R1) was grown in the presence of total chromium at variable concentrations removed only 10% of chromium from medium containing 1 mg L^{-1} of external metal concentrations after 12 days of incubation with further decline in removal efficiency at 5 mg L^{-1} (Fig. 12), indicating poor chromium removal ability by live cells. Interestingly, there was an increase in chromium removal efficiency by 44-67% from medium containing 10 - 20 mg L^{-1} of total Cr, however there was concomitant decrease in cell count (Fig. 12). The increase in metal sorption suggest shift in the mechanism of metal removal from bioaccumulation to biosorption, since at higher metal concentration due to metal toxicity the dead cells exhibited an enhanced metal removal along with metal resistant cells.

Table 11. Effect of total Cr concentration on metal removal and growth of *Chlorella* sp. (R1).

Parameter Analysed	Total Cr (mg L^{-1})			
	1	5	10	20
Metal removal efficiency (%)	9.8	6.8	66.6	44.2
Metal uptake (mg g^{-1})	-	31.92	47.5	60.7
Chlorophyll x 10^{-4} (mg ml^{-1})	21.68 ± 0.0009	0.84 ± 0.000005	1.5 ± 0.000006	-
Microalgal cell (Count x 10^5 ml^{-1})	93.6	5.3	4.8	2.5

The specific metal uptake (q) for total Cr increased concomitantly with increase in metal concentration in medium with maximum metal uptake (q_{max}) of 60.70 mg g^{-1} of as calculated by mass balance for *Chlorella* sp. (R1) biomass (Table 11). Thus with the above study dead biomass of *Chlorella* sp. (R1) isolated from wastewater has gained an attention as a potential

candidate for chromium remediation from wastewater stream and further research in this area is warranted.

2.2 Metal removal potential of microalgal consortium (CP1) in single metal solution

The microalgal consortium (CP1) which was developed from pond wastewater and consisting of a mixed culture of *Chlorella* sp. >*Chlamydomonas* >*Lyngbya* sp. was found to remove appreciable amount of heavy metal during its growth. The metal removal ability was maximum for Zn^{2+} followed by total Cr and Pb^{2+} as observed from extensive single metal removal study carried out under batch conditions under variable initial metal concentration. The results showing effect of each metal concentration on growth, metal removal and uptake has been explained as below.

2.2.1 Effect of varying Zn^{2+} concentration on growth and its removal

The effect of varying Zn^{2+} concentration was studied on growth and metal removal by microalgal-consortium (CP1) developed from wastewater of wastewater stabilization pond oxidation pond under batch conditions using live biomass. Zn^{2+} demonstrated its direct toxic effect on growth of consortium (CP1) as observed in terms of proportional decline in chlorophyll content with increase in Zn^{2+} concentration. A significant reduction by 88% of chlorophyll content was observed in medium containing 10 mg L^{-1} of Zn^{2+} over its nonmetallic control which was further declined by increasing external metal concentration in the medium (Fig. 13). Despite of the toxic effect of Zn^{2+} on chlorophyll which act as an indicator of growth and metabolism, consortium (CP1) revealed its maximum ability for removing Zn^{2+} from BG-11 medium during its 15 days of growth, which varied from 68-73% at an optimum external Zn^{2+} concentration of 1-20 mg L^{-1}. Both microalgal consortium (CP1) and pure culture of *Chlorella* sp. (R1) had significant metal uptake potential. Maximum metal uptake capacity (q_{max}) of 63.92 mg g^{-1} was calculated for Zn^{2+} by mass balance for live microalgal consortium (CP1) (Fig. 13).

2.2.2 Effect of varying Pb^{2+} concentration on growth and its removal

End point chlorophyll was measured as an indicator of microalgal growth after 15 days of incubation for each metal containing medium, which showed toxic effect of increasing metal concentration by Pb^{2+}. Maximum chlorophyll value of 6.9 x 10^{-4} mg ml^{-1} was observed for

CP1 in media containing ≤ 20 mg L^{-1} of Pb^{2+} followed by marked reductions in chlorophyll content by 97 % for CP1 was observed by increasing Pb^{2+} concentration from 10 to 100 mg L^{-1} in medium, where measurement of chlorophyll content acted as a key indicator of microalgal cells metabolism and growth (Fig. 14). In presence of 10 mg L^{-1} of Pb^{2+} there was stimulation in the growth of consortium as chlorophyll content increased from 3 x 10^{-4} to 6.9 x 10^{-4} mg ml^{-1}, compared to non metallic control (Fig. 14) whereas drastic reduction of 63.7% in removal efficiency of Pb^{2+} was observed by increasing external Pb^{2+} concentration from 10 to 20 mg L^{-1} which can be directly correlated with a similar trend of reduction in chlorophyll content by increasing Pb^{2+} concentration. A direct correlation of decreasing chlorophyll content with reduction in Pb^{2+} removal efficiency by increasing external Pb^{2+} concentration from 10 to 100 mg L^{-1} was observed (Fig. 14). Microalgal consortium (CP1) consisting of a mixed culture of *Chlorella* sp. >*Chlamydomonas* >*Lyngbya* sp. was found to remove 17% of Pb^{2+} after 15 days of incubation from culture media containing 10 mg L^{-1} of Pb^{2+}, which decreased by increasing metal concentration from 20-50 mg L^{-1} (Fig. 14). A drastic reduction of 63.7% in removal efficiency of Pb^{2+} was observed by increasing external Pb^{2+} concentration from 10 to 20 mg L^{-1}. Despite of low level of lead removal by consortium, an increase in Pb^{2+} uptake was observed by increasing external metal concentration as calculated from solution mass balance, where maximum uptake capacity (q_{max}) of 33.31 mg g^{-1} was observed (Fig. 14).

2.2.3 Effect of varying total Cr concentration on growth and its removal

Chromium was observed as more toxic than both Pb^{2+} than Zn^{2+} to microalgal chlorophyll with least susceptibility for removal by live biomass. Chromium demonstrated its metal toxicity to microalgal consortium (CP1) by inhibiting its growth even at 1 mg L^{-1} as indicated decline in chlorophyll content over its non-metallic control. This reduction in chlorophyll content continued with further increasing external metal concentration till 50 mg L^{-1} where 75% reduction in chlorophyll content was observed by further increasing total Cr from 1 to 5 mg L^{-1}. In batch study where consortium (CP1) was grown in the presence of total chromium at variable concentrations removed only 20-25% of chromium from medium containing 1-5 mg L^{-1} of external metal concentration after 12 days of incubation with further decline in removal efficiency to 10 % at 50 mg L^{-1} (Fig. 15), indicating poor chromium removal ability.

2.3 Metal removal potential of Chlorella sp. (R1) in bimetallic condition

Most of the wastewaters to be treated contain high concentration of more than one metal. The effect of three heavy metals (Pb^{2+}, Zn^{2+} and total Cr) was studied on the growth and their removal by *Chlorella* sp. (R1) under bimetallic conditions i.e. Pb^{2+}-Zn^{2+}, Pb^{2+}- Cr(total) and Zn^{2+}-Cr(total) over a concentration range of 1 to 50 mg L^{-1}.

2.3.1 Effect of Zn^{2+} -Pb^{2+} on growth and metal removal by Chlorella sp. (R1)

Since there was precipitation at higher concentration in the medium in the bimetallic system of Zn^{2+}-Pb^{2+}, Zn^{2+} was varied from 1 mg L^{-1} to 45 mg L^{-1} and Pb^{2+} from 1 mg L^{-1} to 20 mg L^{-1}. In bimetallic system due to competitive interactions amongst metals for binding onto metal binding sites and accumulation inside the cell 3D presentation of results revealed effect of presence of one metal on removal of other by *Chlorella* sp. (R1) (Fig. 16A, B, C).

The growth of *Chlorella* sp. (R1) under bimetallic condition showed consistent decrease in cell count with respect to increase in Zn^{2+} concentration whereas, presence of Pb^{2+} enhanced cell number upto 20 mg L^{-1} (Fig. 16A, B). The study revealed metal removal by live *Chlorella* sp. (R1) from bimetallic mixture differs with regard to its ability to interfere with removal of one metal by other metal. A decline in Zn^{2+} removal by 8-15% was observed over its maximum removal efficiency of 83-86% due to the presence of Pb^{2+} as secondary metal whereas, a considerable enhancement in Pb^{2+} removal by 22-23% was observed in the presence of Zn^{2+} as secondary metal and increase with increasing Zn^{2+} concentration (Fig. 16A, B). A maximum metal uptake capacity (q_{max}) of Zn^{2+} and Pb^{2+} were calculated to be 59.68 mg g^{-1} and 121.05 mg g^{-1} from solution containing Zn^{2+} –Pb^{2+}.

2.3.2 Effect of Pb^{2+} -Cr (total) on growth and metal removal by Chlorella sp. (R1)

As the presence of Zn^{2+} enhanced the removal of Pb^{2+} in bimetallic system of Pb^{2+}-Zn^{2+}, the presence of total Cr however suppressed Pb^{2+} removal efficiency in a mixture of Pb^{2+}-Cr(total). *Chlorella* represented as a good candidate for Pb^{2+} removal also even in the presence of total Cr as observed by its high Pb^{2+} removal potential from bimetallic solution containing Pb^{2+} and total Cr (Fig. 17A). A decline in Pb^{2+} removal efficiency of *Chlorella* sp.

(R1) by 5-15% was observed by increasing total Cr concentration, whereas this effect of increasing total Cr was not observed at lower concentration of Pb^{2+} as primary metal. Cr removal capacity of *Chlorella* sp. (R1) varied from 4-10% at total Cr concentration ranging from 1-20 mg L^{-1} and 1 mg L^{-1} of Pb^{2+} (Fig. 17B), which was quite low. Metal uptake capacity (q_{max}) of Pb^{2+} and total Cr were calculated to be 154.46 mg g^{-1} and 232.63 mg g^{-1} respectively from solution containing Pb^{2+} - total Cr.

2.3.3 Effect of Zn^{2+} -Cr (total) on growth and metal removal by Chlorella sp. (R1)

Zn^{2+} removal from culture media containing Zn^{2+} and total Cr showed decline in Zn^{2+} removal efficiency by increasing Zn^{2+} concentration from 1 to 40 mg L^{-1} with an average removal of 60 % upto 10 mg L^{-1} of Zn^{2+} at all concentration of Cr, suggesting least interference of two metals. Cr removal capacity of *Chlorella* sp. (R1) varied from 1-12% at total Cr concentration ranging from 1-20 mg L^{-1} which decreased further by increasing Zn^{2+} concentration (Fig. 18A). Metal uptake capacity (q_{max}) of Zn^{2+} and total Cr were calculated to be 459.73 mg g^{-1} and 137.4 mg g^{-1} from solution containing Zn^{2+}-Cr (total).

3. Development of strategy for wastewater treatment using microalgae

3.1 Indoor studies on wastewater treatment using microalgae

To study the role of microalgae in waste water treatment a time course study was carried on laboratory scale to compare the changes in wastewater characteristics by inoculated microalgae. Experiment was set in four round plastic tubs of 10 L capacity, each filled with 8 L of wastewater prepared after diluting wastewater sample collected from wastewater stabilization ponds at village Sanghol, Distt. Fatehgarh, Punjab, India. with BG-11 medium (half strength) in the ratio of 1:3. Four tubs were divided into two sets of two each, from which tub 1 of each set was kept as control, whereas tub 2 was inoculated with 50 ml log phase culture of *Chlorella* sp. (R1). One set of two tubs was kept in growth room maintained at 28 ± 2 ºC at 3000-3500 lux, light intensity provided by cool white daylight fluorescent lamps where as other set was kept under natural outdoor conditions of light and atmosphere. Representative samples from each tub were drawn at an interval of ten days and characterized for temperature, pH, electrical conductivity, salinity, chemical oxygen demand and five day

biochemical oxygen demand. Loss of water by evaporation was maintained by addition of sterile distilled water.

The experiment was carried out for a period of 80 days starting from the month of August to November where the variation in mid day wastewater temperature was defined by the environmental weather for outdoor tubs, which varied in a range of 10.5 to 29.7°C with the mean temperature of 21.9 and 21.75°C for tub 1 and 2 respectively whereas, this effect was minimized for other set of two tubs by providing controlled optimum conditions for microalgal growth and thus wastewater temperature varied between a minor range of 23.4 to 28.6°C during complete study period (Table 12). The pH of the wastewater was alkaline at the start of experiment and was in range of 8.38 to 8.71 however, it tends to become more alkaline with passage of time, and at the end of 80^{th} day a significant increase in pH was observed for each tub containing wastewater (Table 12). This increase in pH was observed for wastewater of both outdoor and indoor tubs.

The comparative advantage of changing environmental conditions with temperature variation was clearly observed in terms of continuous decline in conductivity and salinity for outdoor tubs. A significant decline in conductivity was observed after 20 days whereas this reduction was not seen for tubs under controlled condition of temperature and light. The role of microalgae was also distinguished in terms of reduction of conductivity and salinity after 20 days of the start of the experiment where wastewater inoculated with *Chlorella* sp. (R1) in tub 2 reduced 60.34% of conductivity whereas this reduction in control tub (tub 1) was 60.78%.

The effect of microalgae and environmental condition was reported on reduction of wastewater COD as observed by comparing COD reduction data of indoor and outdoor tubs for 80 days. There was an increase in COD at 10^{th} day in all the tubs which however showed a continuous reducing trend indicating COD removal in due course of time (Fig. 19A, B). The indoor conditions favourable for the growth of microalgae showed an advantage in terms of COD removal where tub 2 inoculated with *Chlorella* sp. (R1) removed 223.7 mg L^{-1} of COD in comparison to tub 1 (control) where COD removal was 158.7 mg L^{-1} (Fig. 19A, B). This COD removal was calculated to 91.98% and 49.59% for tub 2 and 1 respectively and thus advocated the role of *Chlorella* sp. (R1) in COD removal in tub 2 against indigenously developed microflora in tub 1 (Fig. 19A). Outdoor conditions showed lesser reduction with

185.6 and 153.6 mg L^{-1} of COD removal from tubs 1 and 2 respectively, showing the role of indigenously developed native microalgae in both the tubs and thus suppressing the effect of inoculated *Chlorella* sp. (R1) in tub 2 (Fig. 19B).

BOD_5 was used as a measure of organic pollution in wastewater which was significantly removed in both indoor and outdoor conditions. The indoor tubs showed a BOD_5 removal of 82.8 and 80.9 mg L^{-1} from tub 1 and 2 respectively after 10 days of incubation which was 76.5 and 60 % reduction in comparison to 0 day BOD_5. The BOD_5 was not further reduced for indoor tubs with passage of time besides some minor fluctuations (Fig. 20A) and overall maintained 63.7 and 73.9 mg L^{-1} reduction in BOD_5 till the end of the experiment. Similarly outdoor conditions for algal growth reduced 71 and 70.6% BOD_5 from tub 1 and 2 respectively after 10 days whereas, fluctuation in BOD_5 was observed in outdoor tubs after 10^{th} day which reached maximum on 40^{th} day followed by sharp decline in BOD_5 (Fig. 20B). The above behaviour of BOD_5 shift in outdoor tubs was probably due to effect of changes in environmental temperature and faster evaporation. At the end of the experiment 63.7 and 73.9 mg L^{-1} reduction in BOD_5 was reported from tub 1 and 2 respectively (Fig 20B).

Table 12. Treatment of wastewater by *Chlorella* sp. (R1) under indoor and outdoor conditions.

Parameter	Growth conditions	Treatment	Incubation (Days)							
			0	10	20	40	50	60	70	80
pH	Indoor	Control	8.54	8.35	8.32	9.23	9.51	9.6	9.65	9.6
		Chlorella sp. (R1)	8.57	8.55	8.57	9.32	9.57	9.3	9.42	9.57
	Outdoor	Control	8.62	9.2	9.56	9.67	9.5	9.23	9.52	9.71
		Chlorella sp. (R1)	8.71	9.37	9.56	9.53	9.23	-	9.43	9.32
EC ($mS\ cm^{-1}$)	Indoor	Control	7.07	8.99	7.28	7.93	7.98	7.97	7.92	8.14
		Chlorella sp. (R1)	7.01	8.56	7.12	8.88	8.45	8.23	8.21	7.94
	Outdoor	Control	7.09	7.62	2.78	4.31	10.12	3.72	3.64	2.18
		Chlorella sp. (R1)	7.01	10.9	2.78	4.57	10.12	4.35	3.53	2.02
Temperature °C	Indoor	Control	25.7	25.7	26.8	25.6	25.9	26.3	27.2	28.6
		Chlorella sp. (R1)	26.7	26.7	27.8	26.4	25.9	26.3	27.2	25.6
	Outdoor	Control	28.7	28.5	24.3	19.9	25.5	20.7	16.7	10.9
		Chlorella sp. (R1)	28.7	27.9	24.3	19.9	25.5	20.5	16.7	10.5
BOD_5 ($mg\ L^{-1}$)	Indoor	Control	108.2	25.4	25.7	27.2	29.3	24	29.3	25.3
		Chlorella sp. (R1)	134.2	53.3	69.3	53.1	68.2	30	41.3	40.3
	Outdoor	Control	86.2	25	68.3	103.5	85.3	-	27.8	22.5
		Chlorella sp. (R1)	100.2	29.4	62.5	81.55	62.7	-	23.2	26.3
COD ($mg\ L^{-1}$)	Indoor	Control	320	412.8	281	276	-	189.2	187.7	161.3
		Chlorella sp. (R1)	243.2	428.8	232.2	230.6	-	65.3	64.5	19.5
	Outdoor	Control	249.6	371.2	247.3	123.3	-	120.3	89.2	64
		Chlorella sp. (R1)	268.8	384	272.1	174.3	-	164.2	125.4	115.2

*****The values are average of three consecutive readings

3.2 Metal removal in packed bed columns

3.2.1 Removal of metals from aqueous solution by silica immobilized Chlorella sp. (R1) biomass in packed bed column

Chlorella sp. (R1) was found as one of the dominating microalga having potential advantage of mass cultivation under pond conditions, therefore ability of dead *Chlorella* sp. (R1) biomass to work as biosorbent after immobilization on silica gel was determined for Pb^{2+}, total Cr and Zn^{2+} removal from synthetic metal solution in packed bed column. Pure culture of *Chlorella* sp. (R1) was mass cultivated, dried and immobilized on silica gel to produce sufficient material for column packing. Removal of Pb^{2+}, Zn^{2+} and total Cr from aqueous solution was studied in separate columns each packed with 2 g silica immobilized *Chlorella* sp. (R1) biomass packed in plastic column. Metal solutions (Total Cr 92.6 mg/L, pH 5.7) (Pb 156.6, pH 4.3) (Zn 82.6, pH 5.5) were gravity fed at an average flow rate of 4.3 ml/min through each column. There was 100% removal of total Cr, 96.4% of Pb^{2+}, and 86% Zn^{2+} in the outlet streams (Table 13). The metal concentration increased in outlet solution exhibiting typical 'S' shaped curves for plots of total metal concentration in outlet solution against inlet showing the progressive saturation of biomass (Fig. 21, 22, 23). There was complete saturation of 2 g immobilized *Chlorella* sp. (R1) biomass after passing through the separation columns 40 ml, 42 ml and 35 ml of synthetic solution, containing total Cr, Pb^{2+} and Zn^{2+} respectively. Metal uptake capacity was calculated by performing a mass balance where total metal recovered from the outlet solution was subtracted from metal added to the column till the outlet solution attain same metal concentration as that of inlet solution. Silica immobilized *Chlorella* sp. (R1) biomass showed effective binding of total Cr, Pb^{2+} and Zn^{2+} with metal uptake capacity of 1.07, 5.24 and 1.27 mg g^{-1} respectively as calculated from column performance, which were further calculated for dead *Chlorella* sp. (R1) biomass without considering the contribution of silica which was inert for metal binding (97.5 mg dead *Chlorella* sp. (R1) biomass packed in 1 g silica immobilized biomass) and thus 10.97, 53.74 and 13.02 mg g^{-1} of total Cr, Pb^{2+} and Zn^{2+} respectively were maximum metal uptake potential for dead *Chlorella* sp. (R1) biomass. The uptake potential as calculated for column experiments showed maximum usability of *Chlorella* sp. (R1) biomass for Pb^{2+} followed by total Cr and Zn^{2+} (Table 13).

After complete saturation of each column with metals, the biomass was regenerated by desorbing metal ions with 0.12N HCl, with recovery of 65%, 87% and 58% of total Cr, Pb^{2+} and Zn^{2+} respectively. The biomass was recharged after elution of adsorbed metal and used for subsequent 2-3 cycle of sorption, with 3-5% reduction in metal removal.

Table 13. Metal removal from aqueous solution and uptake by silica immobilized 2g of *Chlorella* sp. (R1) biomass in packed bed column.

Metal	Inlet metal (mg L^{-1})	pH	Flow Rate (ml min^{-1})	Metal removal (%)	Total metal uptake by immobilized biomass (mg g^{-1})	Metal recovery by immobilized biomass (mg g^{-1})	Metal recovery (%)
Pb^{2+}	156.6	4.3	4.1	96	5.24	4.57	87
Total Cr	92.6	5.7	4.3	100	1.07	0.69	65
Zn^{2+}	82.6	5.5	4.3	86	1.27	0.74	58

The effect of increase in flow rate was tested during this study where the above study was performed with columns of reduced bed height by half after packing 1g of biomass in same plastic column with identical dimensions. The columns with reduced biomass quantity of 1 gm per column were fed with respective metal solution of total chromium (Total Cr 75.6, pH 5.5), lead (Pb^{2+} 95.6, pH 4.1) and zinc (Zn^{2+} 70.0, pH 5.5), thus flow rate was increased from an average value of 4.23 to 12.29 ml min^{-1} (Table 13, 14). The results showed a maximum metal removal of 77.54 mg L^{-1} of total Cr whereas these values were 72.48 mg L^{-1} and 58.0 mg L^{-1} for Pb^{2+} and Zn^{2+} respectively as observed from the first 5 ml aliquot collected from outlet stream (Fig. 20, 21, 22). With increase of flow there was a decrease by 22.35%, 25% and 32.55% in total Cr, Pb^{2+} and Zn^{2+} removal efficiency respectively (Table 13, 14). *Chlorella* sp. (R1) biomass also showed less binding for all the three metals with maximum uptake of 5.43, 8.91 and 7.58 mg g^{-1} for Pb^{2+}, total Cr, and Zn^{2+} respectively. The comparison of maximum uptake by *Chlorella* sp. (R1) biomass for total Cr, Pb^{2+} and Zn^{2+} revealed that the column efficiency decreases in proportion to the reduction of bed volume (Table 13, 14).

Table 14. Metal removal from aqueous solution and uptake by silica immobilized 1g of *Chlorella* sp. (R1) biomass in packed bed column.

Metal	Inlet metal (mg L^{-1})	pH	Flow Rate (ml min^{-1})	Metal removal (%)	Total metal uptake by immobilized biomass (mg g^{-1})	Metal recovery by immobilized biomass (mg g^{-1})	Metal recovery (%)
Pb^{2+}	95.6	4.1	12.5	72	0.87	0.73	83.5
Cr Total	75.6	5.5	12.2	77	0.53	0.47	89.0
Zn^{2+}	70.0	5.5	12.2	58	0.75	-	-

3.2.2 Removal of total Cr from chrome electroplating industrial wastewater by silica immobilized Chlorella sp. (R1) in packed bed column

Removal of total Cr from aqueous solution using silica immobilized *Chlorella* sp. (R1) in packed bed column was significant, therefore in order to use such biomass for onsite application removal of total Cr from chrome plating industrial wastewater and its uptake was studied. The electroplating industrial wastewater source contained 682 mg L^{-1} of total Cr and was diluted with distilled water to a concentration of 341 mg L^{-1} of total Cr (pH 1.44) and was gravity fed into the column packed with 2g immobilized *Chlorella* sp. (R1) biomass at a flow rate of 4.14 ml min^{-1}. 80% of total Cr was found to be removed by *Chlorella* sp. (R1) biomass from first 5 ml fraction passed through the column, where 68 mg L^{-1} total Cr was present in collected aliquot (Fig. 27). The concentration of total Cr increased in outlet solution with respect to further passing of wastewater due to progressive saturation of biomass. A complete saturation of 2 g *Chlorella* sp. (R1) biomass was achieved after passing 90 ml of solution as the column outlet solution from biosorption column reaches the same concentration as of inlet wastewater (Fig. 27). The plot of total Cr concentration in outlet solution against inlet volume exhibited the typical 'S' shaped curve for which the total Cr uptake capacity of *Chlorella* sp. (R1) biomass was calculated by performing a mass balance. The total Cr found in 90 ml outlet solution was 26.20 mg and when subtracted from total amount of Cr added 30.69 mg, resulted in a calculated value of 4.48 mg of total Cr adsorbed on 2 g of immobilized biomass. Chromium sorption capacity of *Chlorella* sp. (R1) without considering the contribution of

silica was calculated to be 22.97 mg g^{-1} (97.5 mg *Chlorella* sp. (R1) packed in 1 gm silica immobilized biomass) which represented maximum metal uptake (q_{max}).

After complete saturation of column with total Cr, desorption was done with 0.12N HCl where 80% of desorbed chromium was collected in first 15 ml HCl passed whereas, remaining was collected in subsequent 20 ml aliquot. The chromium desorbed was 3.56 mg, which was 79.46% of the 4.48 mg of total metal sorbed (Table 15).

The effect of increase in flow rate was also tested during this study in same column conditions where the bed height was reduced to half by packing 1g of biomass in same plastic column with identical dimensions. With increase in flow rate from an average value of 4.14 to 12.49 ml min^{-1} (Table 15), there was decrease in total Cr uptake (i.e, adsorbed total Cr) by 24% where only 12.18 mg of total Cr was adsorbed per gram of *Chlorella* sp. (R1) biomass (Fig. 27, 28). This suggests the material is relatively homogeneous and the column packing procedure and subsequent flow characteristics are reproducible.

Table 15. Total Cr removal from industrial wastewater and uptake by silica immobilized *Chlorella* sp. (R1) biomass in packed bed column (Conditions: Initial Cr concentration 341 mg L^{-1}, pH 1.44).

Immobilized Biomass (g)	Flow Rate (ml min^{-1})	Metal removal (%)	Cr recovery by immobilized biomass (mg g^{-1})	Total Cr uptake by *Chlorella* sp. (R1) (mg g^{-1})
1	12.49	56.11	1.95	12.18
2	4.14	80.07	1.78	22.97

3.2.3 Removal of Pb^{2+} from aqueous solution by silica immobilized microalgal consortium (CP1) in packed bed column

The silica immobilized *Chlorella* sp. (R1) showed effective potential for total Cr removal than Pb^{2+} and Zn^{2+} under packed bed condition after immobilization whereas in an another study microalgal consortium (CP1) immobilized in silica showed high removal and uptake of Pb^{2+} under continuous system. In column study, microalgal consortium (CP1) consisting of a mixed culture of *Chlorella sp.>Chlamydomonas sp. >Lyngbya sp.* immobilized in silica gel packed under fixed-bed condition was found to remove appreciable amount of lead from metal solution. Synthetic solution (100 ml) with pH 4.3 and 33.90 mg L^{-1} of lead was gravity

fed to the column and 5 ml fractions of the effluent were collected, acidified and analyzed for Pb^{2+} to yield the plot of residual Pb^{2+} concentration in outlet stream as a function of volume (Fig. 29). A drastic reduction in Pb^{2+} concentration was observed after passing metal solution through the column where 86.81 % Pb^{2+} removal in first 5 ml aliquot and rose to a maximum of 92.5 % removal when 15 ml of synthetic solution was passed through the column. A complete saturation of column was achieved by passing 90 ml of synthetic solution (Fig. 29), for which the adsorption and recovery of Pb^{2+} was assessed by performing a mass balance on Pb^{2+} solution after passing through the column. The total amount of Pb^{2+} found in 90 ml effluent was 1.459 mg and when subtracted from total amount of Pb^{2+} added 3.231mg, resulted in a calculated value of 1.77 mg of Pb^{2+} adsorbed by 0.111 g of dried consortium biomass packed in 1.5 g of immobilized biomass, giving a calculated value of 15.95 mg of Pb^{2+} could be adsorbed by 1 gm of consortium biomass. After complete saturation of biomass with Pb^{2+}, column was washed with 0.12 N HCl recovering 1.52 mg of Pb^{2+} in 50 ml solution, which was calculated to be 86.16 % of adsorbed lead by the column showing its multiple usability. Plot of effluent Pb^{2+} concentration with effluent volume obtained exhibited the typical 'S' shaped curve of a fixed bed-column as the performance of a biosorbent packed column is proportional to the distance of saturation point from the origin of axis. The columns Silica acted as a promising material for immobilization of biomass by offering some advantages such as improved mechanical strength and chemical stability however a decreased flow rate of 0.036 ml min^{-1} was obtained indicating clogging probably due to small particle size of silica.

The study however concludes that dead biomass of same consortium can be immobilized on silica and used under continuous column to remediate metal containing wastewater streams with high metal loading which is cost effective in using a single column for multiple cycles.

DISCUSSION

1. Monitoring of microalgal diversity of wastewater

1.1 Microalgal Diversity

Microalgae emerges as a promising candidate in all forms of natural aquatic treatments of wastewater since they supply molecular oxygen produced as a result of photosynthesis to heterotrophic partners and supporting the initial steps of pollution degradation (Cerniglia, 1992) whereas, nascent oxygen added to the wastewater further oxidize organic pollutants of wastewater. Microalgal community play a number of roles in the biological processes involved in stabilization ponds: (1) they provide conditions to reduce the biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), inorganic nutrients (N and P) and pathogens (coliform bacteria) and at certain densities, they keep the aerobic phase facultative pond functional (Barrera *et al*., 2008). This processes in ponds has been referred to as turning "sewage BOD" to "algal BOD", in a cooperative manner between bacteria and microalgae (Mara and Pearson, 1998). In the present periodic study, the effect of seasonal variation was studied on microalgal dynamics of facultative pond wastewater (Table 5). Nine genera of microalgae were reported belonging to three phylum (Cyanophyta, Chlorophyta and Bacillariophyta) from which highest number of genera corresponded to Chlorophyta (*Chlorella* sp., *Chlorococcum* sp., *Closteriopsis* sp. and *Chlamydomonas* sp.) (Tharavathi and Hosetti, 2003) followed by Cyanophyta (*Nostoc* sp., *Lyngbya* sp., *Myxosarcina* sp., *Gloeocapsa* sp.,) and Bacillariophyta (*Diatoms*) (Table 4). Microalgae identified in our study have been previously described in other wastewater stabilization pond systems (Mendes *et al*., 1995; Kirkwood *et al*., 2003; Tharavathi and Hosetti, 2003; Martyn *et al*., 2004; Ahmadi *et al*., 2005; Escorihuela *et al*., 2007; Barrera *et al*., 2008; Shanthala *et al*., 2008). Chlorophyta were found as a dominant contributor of microalgal community, since these microalgae are able to withstand the broad variations in physico-chemical parameters that occur in this system (Roche 1995). Cyanophyta were second dominant group in pond system since they can grow under oxygen limiting conditions (Martyn *et al*., 2004).

Chlorella was reported as a predominant genus group present in facultative pond wastewater and observed in abundance (Tharavathi and Hosetti, 2003; Shanthala *et al.*, 2008). Due to these microalgae facultative ponds takes a dark green color, although they may

occasionally appear red or pink (especially when slightly overloaded) due to the presence of anaerobic purple sulphide-oxidizing photosynthetic bacteria. The other microalgae that tend to predominate in the turbid waters of facultative ponds are the motile genera (such as *Chlamydomonas*, *Pyrobotrys* and *Euglena*) as these can optimize their vertical position in the pond water column in relation to incident light intensity and temperature more easily than non-motile forms (such as *Chlorella*, although this is most common in facultative ponds) (Mara, 1997).

High concentration of organic matter in tropical oxidation ponds supported the development of Cyanophyta (Kumar, 2002) the second dominant group. The Cyanophyta dominance from February to May was confirmed with the observation of Kumar (2002) who observed the formation of algal blooms by blue green algae. The important factor responsible for the formation of a blue Fgreen algal bloom are increased oxidisable organic matter, CO_2, phosphate and calcium (Ramaswamy and Somashekar, 1982). The genera belonging to class Bacillariophyta was reported only in the month of December whereas, it was totally absent during the months from January to May and then from August to November with similar observation by Shanthala and his co-workers (2008) who have also observed their maximum growth in winters from the months of October to January whereas, minimum in February to May due to their inability to sustain higher temperature and they might have produced autotoxin resulting in their abrupt disappearance.

Changes of pH, temperature and light intensity control the abundance and activity of specific groups of microorganisms in the multi-species microbial communities' characteristic of facultative ponds (Murakani *et al.*, 1992; Wilderer *et al*., 1991). *Euglena variabilis*, *Chlamydomonas reinhardii* were the most dominant types of green algae that were indication for high organic load (Mara and Pearson, 1998). *Euglena variables* and *Chlamydomonas reinhardii* were predominant in anaerobic, facultative and maturation effluents however, bioactivity of a complex waste is probably related to interactions among components with no substance having dominant effect (Walsh *et al.*, 1980). High algal diversity also results in efficient nutrient removal from primary settled wastewater in facultative pond (Lau *et al*., 1992).

1.2 Characterization of wastewater and treatment

1.2.1 Wastewater stabilization pond for wastewater treatment

Onsite case study was conducted on pond system at village Sanghol, Distt. Fatehgarh Sahib, Punjab, India, to study microalgal diversity and its role in wastewater treatment. The domestic wastewater from entire village was fed into anaerobic pond (Equalization tank) where treatment of wastewater by anaerobic bacteria releases methane and carbon dioxide (Bitton, 2005). The case study was focused on the performance of facultative pond which receives wastewater after passing through anaerobic pond and mainly aimed to reduce the pollutants to desired levels for its end use in irrigation or fish culturing in maturation ponds. The inlet and outlet wastewater samples of facultative ponds were compared for wastewater characteristics to determine treatment potential of microalgal diversity (Table 6, 7).

1.2.2 Temperature

The pond inlet wastewater temperature varied with respect to seasonal variations from a minimum of 12.7 °C in the month of January to a maximum of 32°C in the month of August. The efficiency of Wastewater stabilization ponds was optimum in hotter climates and reduced in cold climate due to poor light availability and low temperature (Gronlund, 2004). However, feasibility of using certain microalgae with special attributes to treat wastewater in cold regions have been demonstrated (Gronlund, 2004). A slight higher temperature of facultative pond outlet water than inlet wastewater stream by 0.2 to 1°C was due to microbial metabolic activities and growth of microalgae (Awuah, 2006).

1.2.3 Conductivity and salinity

An average 47% and 48% reduction in conductivity and salinity was observed after treatment respectively. The electrical conductivity (EC) of a soil or water is influenced by the concentration and composition of dissolved salts where salts increases the ability of a solution to conduct an electrical current, so a high EC value indicates a high salinity level (Ensink *et al*., 2007). Salinity (expressed as electrical conductivity) of inlet wastewater varied from 1.94-4.67 mS cm^{-1} with a mean of 3.63 mS cm^{-1} which was reduced by an average of 47% over complete observation period with outlet water maintaining salinity of minimum 1.75 mS cm^{-1}

to maximum 2.12 mS cm^{-1} with a mean of 1.92 mS cm^{-1} over the year (Table 7). Salinity act as an important parameter if the treated water is to be used for crop irrigation (Rhoades *et al*., 1992). The most commonly irrigated crops in Fatehgarh sahib area of Punjab is wheat and cotton which have a relatively high salt tolerance with 6.0 mS cm^{-1} and 7.7 mS cm^{-1} respectively, thus this pond treated water is recommended for irrigation to such crops on the basis of its salinity whereas, it can be used after adition of fresh water for crops like rice (3.0 mS cm^{-1}), cauliflower (1.8 mS cm^{-1}), spinach (3.2 mS cm^{-1}) and tomatoes (0.9 mS cm^{-1}) which are more sensitive to salinity (Rhoades *et al*., 1992). It has also been observed that salinity of outlet wastewater from facultative pond was higher in summers than winters which may be due to the enhanced evaporation of water from pond system. Ensink and his co-workers (2007) observed 8,750 m^3 d^{-1} of water loss by evaporation from the facultative and maturation pond which was 40% of daily inflow and the cause of 67% increase in salinity of the effluent (Ensink *et al*., 2007).

1.2.4 Dissolved oxygen and pH

The dissolved oxygen (D.O.) and pH act as indicators of water treatment and plays an important role if the treated water is to be used for irrigation or fish culturing (Phan-Vana *et al*., 2008). pH and dissolved oxygen (D.O.) of the facultative pond was found to increase as a result of wastewater treatment (Table 6, 7) The increase in pH were comparable with previous reports, an increase from 7.7 to 8.5 was observed after treatment and believed to be associated with CO_2 consumed during microalgal photosynthesis (Chisti, 2008). In a similar study conducted on microalgal based ponds an increase in (D.O.) from 5.3 to 8.7 after treatment was observed (Msuya and Neori, 2002).

1.2.5 Chemical oxygen demand and Biochemical oxygen demand

The COD and BOD_5 are used as an indicator of chemical and organic pollutions in the wastewater showed marked variation in inlet wastewater (Table 6, 7) whereas, removal of COD and BOD_5 in facultative pond outlet water showed pond treatment potential. The role of microalgae is well credited for maximum COD and BOD5 removal especially in facultative and maturation ponds as the microalgae utilise inorganic nutrient for their growth by lowering COD and produce nascent oxygen during photosynthesis which augments complete oxidation

of organic compounds resulting in lowering of BOD_5 (Cerniglia, 1992). On an average 41 to 93% reduction in COD and 53 to 79% reduction in BOD_5 was observed during the study period, which was comparable with the latest study carried out by El-Deeb Ghazy and El-Senousy (2008) for performance evaluation of a waste stabilization pond in a rural area in Egypt and observed COD and BOD_5 removal of 20.24% and 21.1% respectively in the outlet of facultative pond. The outlet wastewater had COD reduced to 80.0-281.6 mg L^{-1} (Mean= 134.6 mg L^{-1}, 50.26 removal), while the BOD_5 was reduced to 20.0-114.2 (Mean= 57.3 mg L^{-1}, 69.10 removal) which was comparable to the treatment potential of waste stabilization pond (WSP) model of domestic wastewater treatment unit in rural area and effluents discharged in the drain had BOD_5 reduced to 109-245 mg L (Mean = 145.3 mg L^{-1}, 50.65% removal), while the COD was reduced to 221-400 mg L (Mean = 289 mg L^{-1}, 48.95% removal) El-Deeb Ghazy and El-Senousy (2008). The COD and BOD_5 concentrations in outlet wastewater fluctuated greatly but no distinguished effect of hot and cold conditions was observed. The target effluent standard of 20 mg L^{-1} for BOD_5 and 50 mg L^{-1} for COD as fixed by WHO (Mara *et al*., 1992) were not achieved except in the month of May (BOD = 20 mg L^{-1}) which can be correlated with maximum growth of green algae in this month. During monsoon (August, September and October) COD and BOD_5 of treated wastewater was 280 and 114 mg L^{-1} respectively, which were high mainly due to increase in the drainage of dissolved rural waste. A similar trend has been observed in refinery ponds where primary productivity during monsoon season was decreased due to the dilution of water and the dispersion of microalgal cells along the system (Nair *et al*., 1988). The pollutant removal efficiency of algal based pond systems has been shown to vary widely and it depends on retention time, water depth, initial nutrient concentration, microflora and harvesting regimes (Nhapi *et al*., 2003).

Another important parameter which effect COD and BOD_5 removal is COD/BOD_5 ratio of inlet wastewater which varied from 0.76 to 7 in ten month observation period whereas it was less than 2 in winter season. The present study showed a COD/BOD_5 ratio in the range from 0.76 to 7, indicates that the wastewater is subject to maximum degradation in winters with COD/BOD_5 value of less than 2 whereas it is moderately biodegradable in summers season. A COD/BOD_5 value depends on the nature of the wastewater such as whether it is municipal or industrial oriented and vary considerably with the degree of treatment the wastewater has undergone (Papadopoulos *et al*., 2001). The COD/BOD_5 ratio value for

municipal raw wastewater is in the range of 1.25 to 2.5, whereas for industrial wastewater is up to 10 or more (Markantonatos, 1990). The municipal wastewater is more biologically degradable than industrial wastewater.

1.2.6 Removal of heavy metals (Total Cr, Pb^{2+} and Zn^{2+})

The inlet wastewater to the pond system was domestic wastewater and the reported heavy metals (Pb^{2+}, Zn^{2+} and Total Cr) were in the range of permissible limit set by Punjab pollution control Board. However, 72% removal of Zn^{2+} and 73% removal of Pb^{2+} was reported in the months of November and March respectively with 28 to 72% removal of total Cr. Uptake of metals adsorption or either due to bioaccumulation by pond microalgae (Soniassy and lemon, 1986) and bacterial sedimentation (Shilton, 2006) has been shown to be an important process for heavy metal removal. On fate of heavy metals in WSP and HRAP little work has been done (Kaplan *et al*., 1987; Toumi *et al*., 2000). Toumi and his co-workers (2000) reported zinc (Zn), copper (Cu) and lead (Pb) removal in waste stabilization pond (WSP) which reached 91, 92 and 71% respectively whereas, microalgae of facultative pond was credited for the appreciable removal of Cu, Cd, Hg, Ni, Pb and Zn (Soniassy and Lemon, 1986). Among the organisms exposed to metal pollution in the lake or pond water, the most dominating phytoplankton like *Chlorella vulgaris* is the most affected and is a potential candidate for metal bioremediation studies (Tharavathi and Hosetti, 2003; Shanthala *et al*., 2008). A linkage establishes between microalgae present in wastewater stabilization ponds and its potential of treating wastewater in terms of removal of organic and inorganic pollutants and heavy metal remediation.

2. Removal of metal (Zn^{2+}, Pb^{2+} and total Cr) by selected microalgae

2.1 Selection of Chlorella sp. (R1) and Algal consortium (CP1) for metal removal studies

The research targeted to remove heavy metals in WSP required the selection and application of desired microalgae in pond system where inherited metal accumulation capability of microalgae could be used to alleviate the burden of toxic metal and to recover precious metals (e.g., gold and silver), though very little research has targeted the fate of heavy metals in WSP and HRAP (Kaplan *et al.*, 1987; Toumi *et al.*, 2000). The preliminary study carried out with the aim of selection of microalgal candidate for Pb^{2+}, Zn^{2+} and total Cr removal from

wastewater advocated the use of *Chlorella* sp. (R1) and microalgal consortium (CP1). The *Chlorella* sp. has reported as a predominant genus group present in facultative pond wastewater (Tharavathi and Hosetti, 2003; Shanthala *et al.*, 2008) and removed Pb^{2+} and Zn^{2+} by 62% and 67% respectively from medium containing 5 mg L^{-1} of external metal concentration. Similarly the microalgal consortium developed from wastewater and which represented indigenously developed microalgae from wastewater dominated by *Chlorella* sp. > *Chlamydomonas* sp. > *Lyngbya* sp. showed effective Zn^{2+} removal potential by removing 68% metal ions from medium containing 5 mg L^{-1} of Zn^{2+}. Among the organisms exposed to metal pollution in the lake or pond water, phytoplankton like *Chlorella vulgaris* are the most affected and thus, it can be a potential candidate for metal bioremediation studies (Rachlin and Grosso, 1993) whereas, system consisting of several micro-organisms is preferably in bioremediation processes since it is nearly impossible to find a microorganism species that can degrade a mixture of different pollutants completely by itself and thus making study of consortium warranted (Alexander, 1994).

2.2 Effect of metal (Zn^{2+}, Pb^{+2} and total Cr) on growth and metal removal by Chlorella sp. (R1) and algal consortium (CP1)

This green alga *Chlorella* has been well studied for various metabolic and stress investigations because of its small size, fast growth rate, and similarity to plants higher in terms of physiology and biochemistry (Rachlin and Grosso 1993). In the study to monitor the effect of varying concentration of metals (Pb^{2+}, Zn^{2+} and Total Cr) on *Chlorella* sp. (R1) growth presented total Cr as most toxic with 50% lethal concentration of 4.34 mg L^{-1}, followed by Zn^{2+} with 10.25 mg L^{-1} whereas Pb^{2+} increased cell division up to 20 m L^{-1}. The pond isolated *Chlorella* sp. (R1) was found more resistant to Cr than previously reported *Chlorella pyrenoidosa* from the culture collection of the University of Tokyo with LC_{50} of 2 mg L^{-1} whereas in other report cell division was suppressed 50% in *C. vulgaris* cultures by Zn^{2+} 5.1 mg L^{-1} (Rosko and Rachlin. 1977; Zsolt *et al.*, 2006;). The detrimental effect of chromium and other cells parts via a Fenton-type mechanism and it also has the capacity to reduce the activity of antioxidant enzymes (Shi and Dalal, 1990; Panda and Choudhary, 2005) however, for Zn^{2+} the growth inhibition may be related to extracellular zinc concentration (Wilde *et al.*, 2006). The increase in cell number indicated no signs of Pb^{2+} toxicity on

Chlorella sp. (R1) up to 20 mg L^{-1} whereas reduction in chlorophyll was observed at 10 mg L^{-1} Pb^{2+} in the medium which supported the previous finding where cell division was suppressed 50% in *C. vulgaris* cultures by Pb^{2+} greater than 100 mg L^{-1} while Pb^{2+} 32 mg L^{-1} actually decreased chlorophyll a content (Rosko and Rachlin, 1977). The Protective role of *Chlorella* against lead toxicity has been found where *Chlorella* has protective effect against lead-induced hepatic toxicity in rats. This study was supported by Daesang Co. Ltd. WelLife Health Business (Jang *et al.*, 2008).

The pure culture of *Chlorella* sp. (R1) studied for its Pb^{2+}, Zn^{2+} and total Cr removal over a varying concentration range maintained in culture medium showed high removal efficiency for Zn^{2+} followed by Pb^{2+} and total Cr. The maximum 60-70% removal of Zn^{2+} was observed from culture medium containing 5-20 mg L^{-1} of external metal concentration whereas Pb^{2+} was maximally removed to 66% from culture medium containing 1 mg L^{-1} and 45 to 50% removal efficiency was maintained for 5 to 20 mg L^{-1} whereas it removed 10% of chromium from medium containing 1 mg/L of external metal concentrations after 12 days of incubation with decline in removal efficiency at 5 mg/L. The Pb^{2+} removal by *Chlorella* sp. (R1) was higher as compared to 32.15% and 46.01% by two *Chlorella* strains WB and SB respectively isolated from Laguna de Bay, Philippines (Nacorda *et al.*, 2007) whereas similar to the findings of Nacorda and his co-workers (2007) in the case of total Cr removal, who observed only 20.8 to 28% metal removal by two isolated strains of *Chlorella* from BG-11 medium containing 1.0 mg/L of Cr. The increase in chromium removal efficiency by 44-67% from medium containing 10 - 20 mg/L of total Cr, with concomitant decrease in cell count suggest shift in the mechanism of metal removal from bioaccumulation to biosorption, since at higher metal concentration due to metal toxicity the dead cells exhibited an enhanced metal removal along with metal resistant cells.

The maximum metal uptake (q_{max}) of 41.75, 34.36 and 60.70 mg g^{-1} for Zn^{2+}, Pb^{2+} and total Cr respectively for live *Chlorella* biomass were reported better for Pb^{2+} and Zn^{2+}, than reported by Sandau and his co-workers (1996) who observed 17.2 and 6.60 mg g^{-1} of q_{max} for Pb^{2+} and Zn^{2+} whereas reported similar to the uptake potential of *Chlorella* biomass as observed in the latest finding of Akhtar and his co-worker (2008) who observed 58.80 mg of Cr^{3+} adsorbed per gram of biomass.

The other culture of microalgal consortium (CP1) consisting of a mixed algae *Chlorella* sp. >*Chlamydomonas* sp. >*Lyngbya* sp. and which represent the indigenously developed microalgae from wastewater was studied for the effect of varying metal concentration (Zn^{2+}, Pb^{2+} and total Cr) on its growth and metal removal potential. Total Cr was observed as more toxic than both Pb^{2+} and Zn^{2+} to algal chlorophyll with least susceptibility for removal by live biomass which showed decline in chlorophyll as its toxic effect even at 1 mg L^{-1}. Microalgal consortium (CP1) had maximum removal ability for Zn^{2+} followed by total Cr and Pb^{2+} as observed from extensive single metal removal study carried out under variable initial metal concentration. Inhibitory effect of Pb^{2+} on microalgal consortium (CP1) growth which was not observed for *Chlorella* sp. (R1) can be discussed by direct toxic effect of lead to the chloroplast as it interact directly with the thylakoid membranes of the chloroplast (Szalontai *et al.*, 1999). Live consortium under study dominated by *Chlorella* sp. (R1) however showed lesser Pb^{2+} removal efficiencies as compared to pure culture of *Chlorella* sp. (R1) reported earlier (Nacorda *et al.*, 2007), this reduction in removal efficiency by mix culture of consortium can be due to the presence of gelatinous capsule around *Lyngbya* found in consortium, which may restrict the diffusion of metal ions into chelating matrix of *Chlorella* sp. (R1) and other contributing algal cell walls (De Philippis *et al.*, 2003). Despite of low level of lead removal by consortium, an increase in Pb^{2+} uptake was observed by increasing external metal concentration as calculated from solution mass balance, where maximum uptake capacity of 33.31 mg/g was observed. Removal of Pb^{2+} by microalgal consortium (CP1) under batch conditions with maximum 17% removal efficiency and 33.31 mg/g as maximum lead uptake capacity demonstrates a good assessment of metal remediation potential of natural waste stabilization ponds containing low concentration of heavy metals by microalgae (Moshe, 1972).

2.3 Metal removal potential of Chlorella sp. (R1) in bimetallic condition

Most of the industrial and domestic wastewater contains high levels of more than one metal (Toumi *et al*., 2000; Lee *et al*., 2004). While the removal of single species of heavy metal ions by algal biomass has been extensively studied, very little attention has been given to the study of bimetallic system. The metal removal study under bimetallic condition with *Chlorella* sp. (R1) showed interaction amongst metal which varied from competition between metals and

thus resulting in decrease in removal efficiency of other metals whereas, enhanced removal of one metal due to the presence other was also monitored. In the bimetallic system of Zn^{2+}-Pb^{2+}, Pb^{2+}-Cr (total), Zn^{2+}-Cr(total) have shown competitive interactions amongst metals for binding onto metal binding sites and accumulation inside the cell where growth results of *Chlorella* sp. (R1) strengthen the findings of single metal system where total Cr is most toxic to *Chlorella* sp. (R1) cell number followed by Zn^{2+} with consistent decrease in cell count with respect to increase in Zn^{2+} concentration whereas, Pb^{2+} was non toxic to cell number till 20 mg L^{-1}. The presence of Zn^{2+} and total Cr enhanced the removal of Pb^{2+} in bimetallic system and thus supported clear shift of dominant metal removal mechanisms from live cells accumulation to dead cells sorption whereas a bit decrease in Pb^{2+} and Zn^{2+} removal efficiency was observed by increasing total Cr concentration as a result of chromium toxicity to *Chlorella* sp. (R1). Same poor total Cr removal capacity of *Chlorella* sp. (R1) was also monitored from bimetallic mixture containing total Cr with Zn^{2+} and Pb^{2+} whereas chromium removal varied from 4-12% at total Cr concentration ranging from 1-20 mg L^{-1}. There is no similar report available in favor of these observation but it has been previously reported that the presence of a other metals leads to interactive effects on physiological and biochemical processes, e.g., growth, metal uptake, etc of various algae used for bioremediation (Ting *et al.*, 1991).

The metal uptake capacity of *Chlorella* sp. (R1) increased initially for one metal by increasing its external metal concentration which was also observed for single metal system whereas, presence of secondary metal e.g., Zn^{2+} and total Cr increased metal uptake of primary metal as a result of increase in toxicity of the solution and shifting removal mechanism from live cell accumulation to dead cell biosorption which showed more metal uptake potential for *Chlorella* sp. (R1). The results were supported by previous findings where metal uptake initially increased with increase in metal concentration in the solution, and then becoming saturated after a certain concentration of metal (Da Costa and Leite, 1991; Aloysius and Arif, 1999; Mehta and Gaur, 2001a,b,c; Mehta *et al*., 2002).

3. **Development of strategy for wastewater treatment using microalgae**

3.1 Indoor studies on wastewater treatment using microalgae

The wastewater demonstrated anaerobic phase where the increase in oxygen was indicated by the rise in pH as observed from wastewater samples with passage of time (Roche 1995; Martyn *et al*., 2004, Kayombo *et al*.,, 2002). From the study conducted under controlled laboratory condition, the role of microalgae especially *Chlorella* sp. (R1) was confirmed as a dominant contributor in removal of organic and inorganic pollutants. Effective contribution of *Chlorella* sp. (R1) in reduction of COD in tubs placed under indoor conditions was observed which provided the optimum conditions for the growth of *Chlorella* sp. (R1) whereas, indigenously developed microflora dominated over development of inoculated *Chlorella* sp. (R1) under outdoor conditions and thus decreased COD removal was observed.

Similarly The onset of transition phase on 10^{th} day coincided with maximum removal of BOD_5 from both indoor and outdoor wastewater samples as Barrera and his co-workers (2008) reported four phases of wastewater treatment with microalgae (Phase I), characterized by high BOD, COD, ammonium and faecal coliforms. This was followed by a transition phase (Phase II), which is an initial aerobic phase (Phase III), when water quality improved considerably, and finally an aerobic phase (IV) with a high percent reduction in these parameters and almost complete organic load removal. Thus most of this oxygen for oxidation of pollutants was likely contributed by algal activity from which role of *Chlorella* sp. (R1) was clearly recognized (Tharavathi and Hosetti, 2003).

3.2 Metal removal in packed bed columns

One of the main focus in microalgal biotechnology is on the removal of heavy metal from industrial effluents and wastewater (Mallick, 2002), where the essential requirement of an industrial sorption system is that the sorbent can be utilized as a fixed or expanded bed system. *Chlorella* sp. (R1) and microalgal consortium (CP1) of *Chlorella >Chlamydomonas >Lyngbya sp.* were chosen in our study for development of strategy aiming their use to remediate metals (Zn^{2+}, Pb^{2+} and total Cr) from industrial effluents. The capacity of *Chlorella* sp. (R1) and consortium (CP1) for removal of heavy metal (Pb^{2+}, total Cr and Zn^{2+}) after immobilization on silica was illustrated under continuous column flow conditions. *Chlorella*

sp. (R1) immobilized on silica showed varying metal removal capacity for each heavy metal (total Cr, $Pb^{2+}$and Zn^{2+}) due to different binding capacity for each heavy metal with microalgal biomass. The pH of metal solutions used in column studies using *Chlorella* sp. (R1) were kept in the range of 4-6 as previous reports dictated the dependence of biosorption on solution/wastewater pH and maximum metal removal by dead *Chlorella* sp. was observed at pH 4-6 (Mallick and Rai, 1993). The chrome industrial wastewater used in above study had pH 1.44 and used as such for chromium removal using packed bed column.

The plot of total metal concentration in outlet solution against inlet volume exhibited the typical 'S' shaped curves for all the metals but the performance of a biosorbent packed column is proportional to the distance of saturation point from the origin of axis (Mehta and Gaur, 2005) thus complete saturation of 2 g immobilized *Chlorella* sp. (R1) biomass after passing 42 ml, 40 ml and 35 ml of synthetic solution, containing Pb^{2+}, total Cr, and Zn^{2+} respectively showed better performance of biomass for Pb^{2+} and total Cr than Zn^{2+} removal. The column experiments further showed effective binding of *Chlorella* sp. (R1) with Pb^{2+} as indicated by its maximum metal uptake value of 53.74 mg g^{-1} of biomass. The candidacy of *Chlorella* sp. (R1) for lead removal was supported by the work of Akhtar and his co-workers (2004) who exploited *Chlorella sorokiniana* immobilized on loofa (*Luffa cylindrica*) and observed maximum Pb^{2+} biosorption capacity of 123.67 mg g^{-1} immobilized biomass with > 96% removal from lead solutions containing 10-300 mg L^{-1} of external metal concentration. The available literature also suggests that Pb^{2+} is sorbed maximally compared to other metals in a majority of algal species (Tiem, 2002; Davis *et al*., 2003).

The silica immobilized *Chlorella* sp. (R1) biomass also had effective removal of total Cr with maximum 100% removal from inlet metal synthetic solution (total Cr 92.6 mg L^{-1}, pH 5.7) with uptake potential of 10.97 mg g^{-1} whereas, it also showed capability of removing chromium from effluent generated by electroplating industry containing very high concentration of total Cr. The increase in total chromium uptake by increasing solution metal concentration showed the proportional increase in metal uptake with increase in external metal concentration (Mehta *et al*., 2002a; Mehta *et al*., 2002b). The reported maximum chromium uptake in these studies were comparable with the findings of Dönmez and Aksu (2002) who reported uptake of 33.8 mg g^{-1} of Cr by *Chlorella* biomass in batch study, whereas it was below optimum under continuous conditions since uptake of 69.26 mg g^{-1} of

total Cr has been reported for *Chlorella* immobilized in loofa sponge (Akhtar *et al*., 2008). *Chlorella* sp. (R1) biomass immobilized in silica revealed effective removal of Pb^{2+} and total chromium whereas it was not so effective for Zn^{2+} removal which reflect biochemical difference in cell wall composition and its suitability for specific metal removal. It has been reported that carboxyl, phosphate and amino groups present on the surface of *Chlorella* are the possible sites for total Cr and Pb^{2+} binding (Han, 2006). The use of immobilized biomass provided an advantage of column multiple usability after desorption of metal with 0.12 N HCl whereas percentage recovery of metal ions varied as maximum recovery was attained for Pb^{2+}>total Cr> Zn^{2+}. Variation in desorption efficiency may be attributed to lesser contact of the desorbant with metal-loaded immobilized biomass (Saeed and Iqbal, 2006). The Silica was found to be inert for metal adsorption (Fig. 21,22, 23), but acted as a promising material for immobilization of *Chlorella* by offering some advantages such as improved mechanical strength, chemical stability and reusability (Mahan and Holcombe, 1992).

A decreased flow rate of 0.036 ml min^{-1} obtained in Pb^{2+} removal by immobilized consortium biomass (CP1) indicated clogging due to small particle size of silica gel used, whereas packed-bed reactor was optimized for operational conditions like pH, flow rate, influent metal concentration, bed height, matrix type, biomass loading and metal concentration prior to large-scale application (Wilkinson *et al*., 1990; Yu and Kaewsarn, 1999; Vijayaraghavan *et al*., 2005).

Chlorella has been extensively explored by many workers for removal of metals e. g Ni, Zn, Cd, Cu, Fe, Hg, Pb, and Au after immobilized in matrix like alginate, agarose, loofa sponge, polyacrylamide, silica gel (Darnall *et al*., 1986; De Costa and Leite, 1991; Awasthi and Rai, 2004; Akhtar *et al*., 2004; Martinez *et al*., 2006) due to its occurrence as a dominating microalga in pond system, whereas a few reports are also available on use of immobilized *Aulosira fertilissima, Scenedesmus* sp., *Navicula canalis* and *Nannochloropsis gaditana* for Zn^{2+}, Pb^{2+} and total Cr removal from wastewater (Travieso *et al*., 1999; Moreno-Garrido *et al*., 2002; Banerjee *et al*., 2004). *Chlorella vulgaris* cells immobilized in silica supplied a good system to remove Pb^{2+}, total Cr and Zn^{2+} from synthetic solution whereas, only a single report is available for using silica immobilize *Chlorella* for Hg removal from wastewater (Martinez *et al*., 2006).

The strategy was aimed at being responsive to the need for improvement of existing treatment processes used for metal removal from industrial and municipal wastewater where the use of bioresins with unique properties seeks to fulfill the need for economically viable recovery of metals for reuse and safe disposal (Brown, 1996). The rationale for the use of biomass derived from microalgae is based on the biological properties of this material where selectivity is based on the polymers present in the cells, particularly in the cell wall. In contrast to the conventional techniques which require high amount of energy input in sludge handling and transport, the technology serves to minimize these needs and is advantageous in terms of process economics (Brown, 1996). *Chlorella* sp. (R1) having small size and allowing for a range of cost-effective cultivation options and growth rates far exceeded those of terrestrial plants whereas, shallow ponds were seen as the most cost-effective way to grow the microalgae (Borowitzka, 1999). In a case study, the cost of operation for treatment of mine site copper contaminated water at Colorado was $6.60 per 1000 gal (Leitz *et al*., 1995) whereas, Darnall estimated operating cost for a bioadsorption plant (using immobilized non-living algae) at $0.25-5.00 per1000 gal, depending on local conditions and process details.

While there are several patents which deal with the approaches of using immobilized microalgae for metal removal, the most relevant of these is a composition patent that used the binding of microbial cells, especially algae, with natural polymers derived from seaweeds, precipitating and heat treating the particles at 300-500°C (Green *et al*., 1991). Another approach has been using a silica gel based composition or immobilization on glass wool (Feiler and Darnall, 1991).

During this laboratory-scale work, microalgal cultures *Chlorella* sp. (R1) and microalgal consortium (CP1) were grown to produce sufficient biomass followed by their immobilization in silica gel. The columns packed with immobilized biomass showed their potential of removing heavy metals (Zn^{2+}, Pb^{2+} and total Cr) from wastewater with proposed application for remediation of industrial wastewater streams with advantages like fixed or expanded bed usability and cost-effectiveness.

CONCLUSIONS

13. Present investigation revealed linkage between microalgae present in wastewater stabilization ponds and its potential of treating wastewater in terms of removal of organic and inorganic pollutants and heavy metal remediation.
14. *Chlorella sp.* (R1) was present during the whole sampling period as one of the most dominating species in pond water followed by *Chlamydomonas*, *Lyngbya, Diatoms*, whereas *Chlorococcum sp*. and *Closteriopsis sp.* were also seen in the month of August and September besides cyanobacteria like *Gloeocapsa* and *Myxosarcina*.
15. Seasonal variations with respect to changes in pH, temperature and light intensity were reflected in abundance and activity of specific group of microalgae in multi-species microbial communities.
16. The pond system showed effective role in wastewater treatment by removing 15 to 83% BOD_5 and 52 to 93% COD, whereas cold climate was found lesser favorable for phycoremediation due to the poor light availability and low temperature.
17. The pond system reduced conductivity and salinity by 47% and 48% respectively of inlet wastewater leading to salinity of 2.12 mS cm^{-1} in outlet water which is good for irrigation purposes.
18. *Chlorella* (R1) had maximum removal potential for Zn^{2+}>Pb^{2+}>Cr (total) whereas Consortium (CP1) had Zn^{2+} > Cr (total) > Pb^{2+} from medium.
19. Removal of metal by *Chlorella* sp. (R1) from bimetallic solutions {Zn^{2+}-Pb^{2+}, Pb^{2+}-Cr (total), Zn^{2+}-Clr(total)} showed interaction among metal ions which resulted in either decrease in removal efficiency or enhancement of removal of one metal in presence of other.
20. Cr was more toxic to *Chlorella* sp. (R1) with EC_{50} of 4.34 mg L^{-1} followed by Zn^{2+} with LC_{50} of 10.25 mg L^{-1} as calculated by probit analysis, whereas Pb^{2+} was nontoxic upto 20 mg L^{-1}.
21. *Chlorella* sp. (R1) was found to have metal uptake capacity (q_{max}) of 34.36, 41.75 and 60.7 mg g^{-1} for Pb^{2+}, Zn^{2+} and total Cr respectively.
22. Efficient contribution of microalgae was observed from microcosm experiments in reduction of COD and BOD_5 with distinct role of *Chlorella* sp. (R1).

23. *Chlorella* (R1) biomass immobilized in silica as supporting material was effective in striping Pb^{2+} from synthetic solution and total Cr from electroplating industrial wastewater.
24. Silica gel was an effective support material for immobilization of algal biomass which provided mechanical strength and chemical stability and this approach can be applied for large scale treatment of industrial wastewater.

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PUBLICATIONS

Research Articles Published

1. Kumar, R and Goyal, D. Domestic Waste water Treatment Using Algae and Duckweeds: A Case Study. Proceedings of the Ninth International In Situ and On-Site Bioremediation Symposium (Baltimore, Maryland; May 7–10, 2007). ISBN 978-1-57477-161-9, published by Battelle Press, Columbus, OH, USA *www.battelle.org/bookstore*.
2. Kumar, R., and Goyal, D. 2009. Comparative removal of Pb^{2+} by live algal consortium and immobilized dead biomass from aqueous solution. *Indian Journal of Experimental Biology*. 46: 690-694.
3. Kumar, R and Goyal, D. 2009. Waste water treatment and metal (Pb^{2+}, Zn^{2+}) removal by microalgal based stabilization pond system. *Indian Journal of Microbiology* .50: 34-40.
4. Kumar, R ., Chaudhary, G., Ahluwalia, S.S and Goyal, D. 2010. Biosorption of Pb^{2+} and Zn^{2+} by non living biomass of *Spirulina*. *Indian Journal of Microbiology*. 50: 4438-442.

Book Chapter

5. Kumar, R and Goyal, D. *Bioremediation of wastewater and role of microalgae* "Algal Biology and Biotechnology" (J.I.S. Khattar, D.P. Singh, Gurpreet Kaur, Eds), I.K. International Publishing House Pvt. Ltd. New Delhi, Bangalore, INDIA. pp 227-250 ik_in@vsnl.net ISBN 978-93-80026-19-0

Paper presented in Conferences

1. Kumar, R* and Goyal, D. Domestic Waste water Treatment Using Algae and Duckweeds: A Case Study. Ninth International In Situ and On-Site Bioremediation Symposium (May 7–10, 2007) Baltimore, Maryland, USA. pp E12.
2. Kumar, R* and Goyal, D. Micro-algal diversity of biological oxidation pond and their role in waste water treatment. Microbial Diversity: Current Perspectives and Potential Applications, University of Delhi South Campus, New Delhi during 16–18 April 2005.

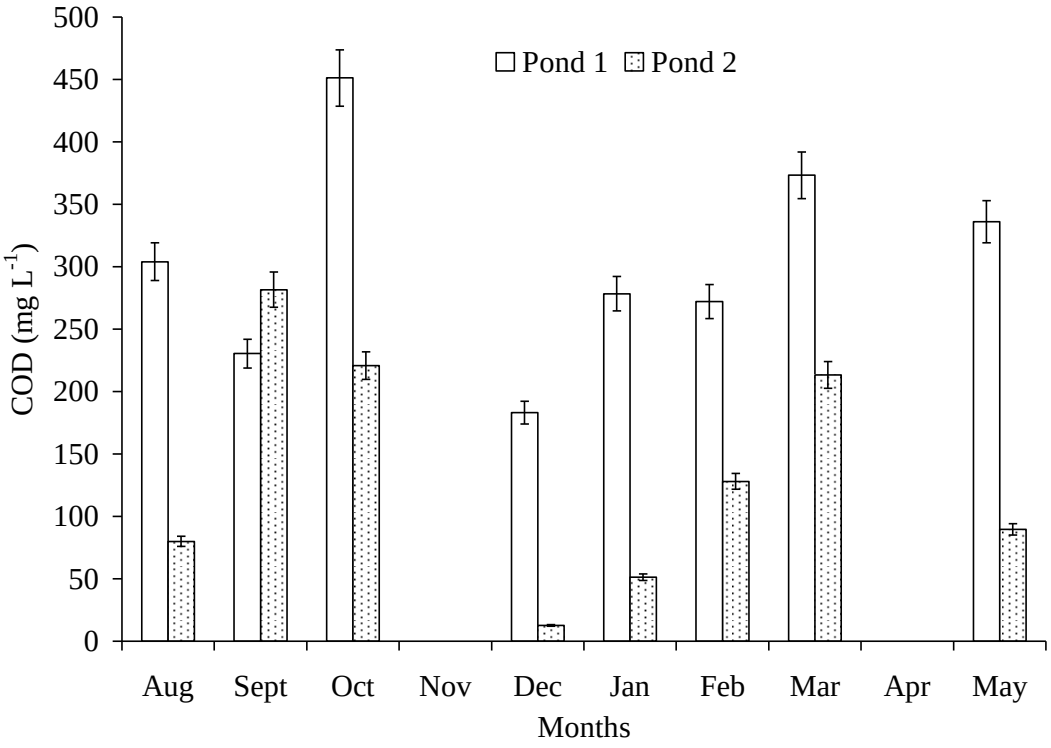


Fig. 4. Seasonal variation in COD of inlet and outlet wastewater.

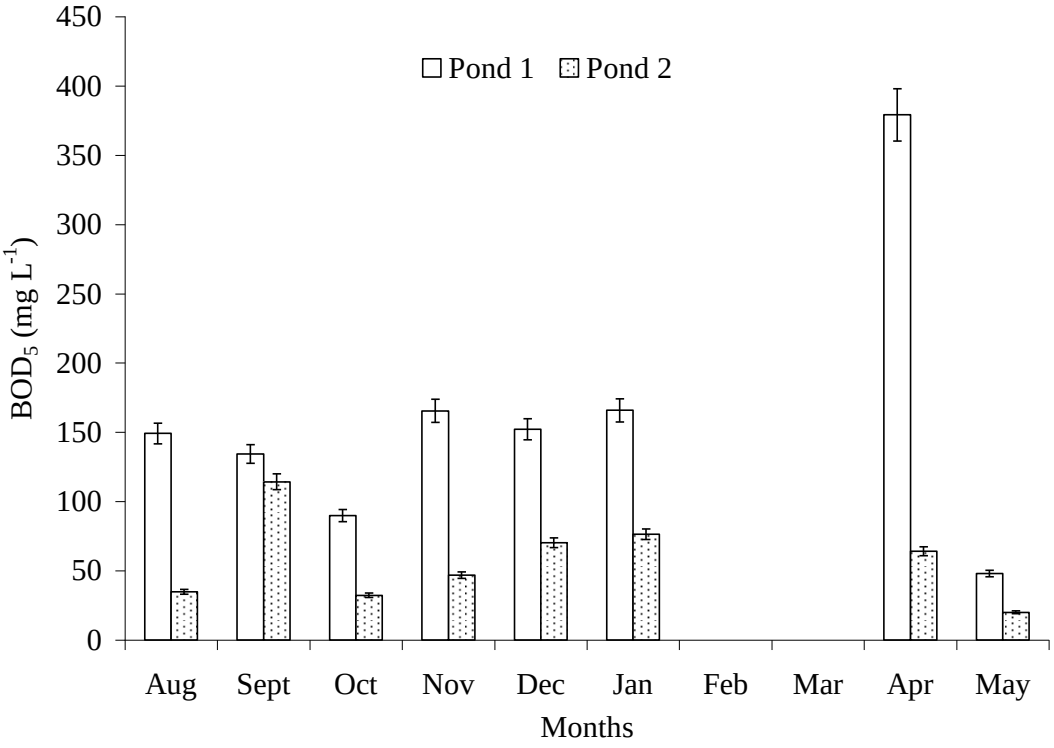


Fig. 5. Seasonal variation in BOD_5 of inlet and outlet wastewater.

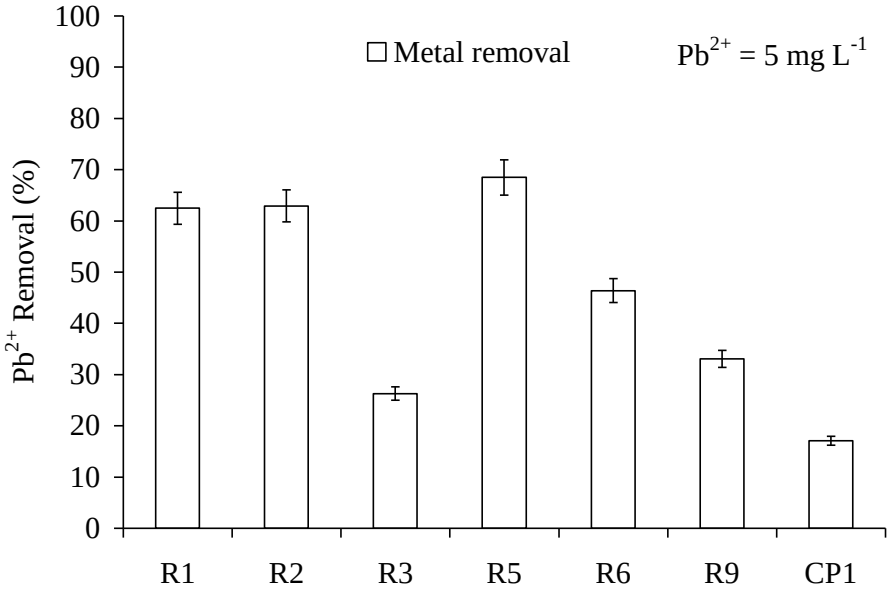


Fig. 6. Removal of Pb^{2+} by unialgal isolates (R1, R2), mixed microalgal cultures (R3, R5, R6, R9) and microalgal consortium (CP1) from aqueous medium after 12 days of incubation at 28 °C, 3000-3500 lux light intensity (Ci = 5 mg L^{-1}).

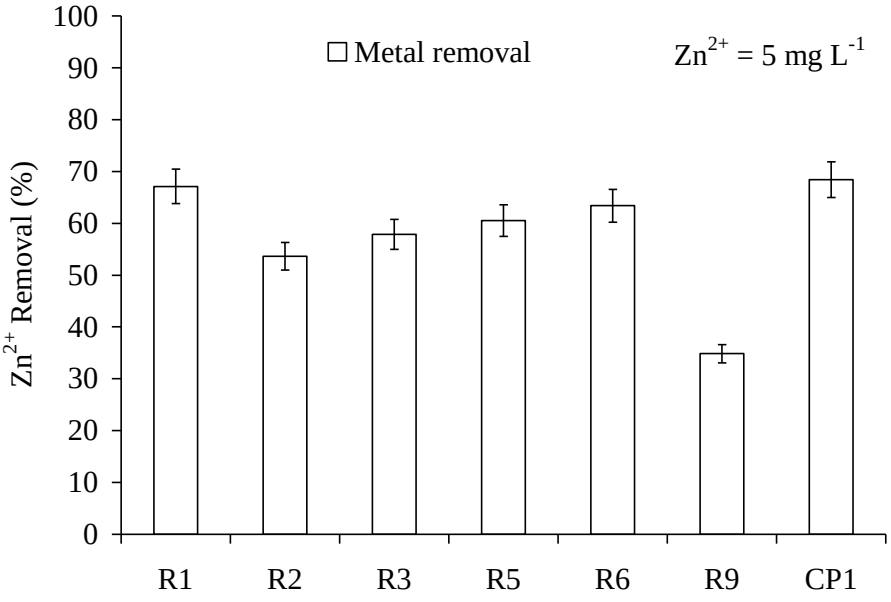


Fig. 7. Removal of Zn^{2+} by unialgal isolates (R1, R2), mixed microalgal cultures (R3, R5, R6, R9) and microalgal consortium (CP1) from aqueous medium after 12 days of incubation at 28 °C, 3000-3500 lux light intensity (Ci = 5 mg L^{-1}).

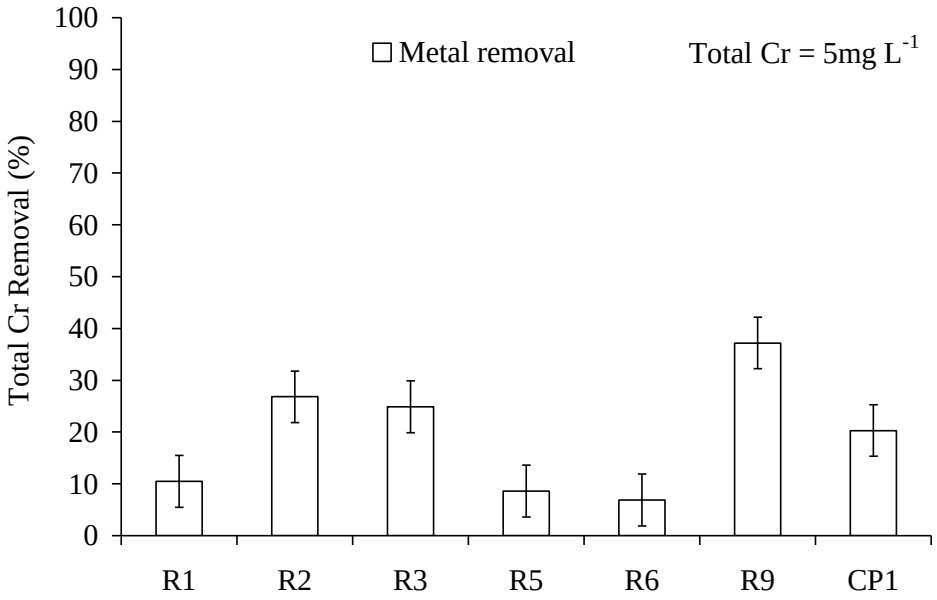


Fig. 8. Removal of total Cr by unialgal isolates (R1, R2), mixed microalgal cultures (R3, R5, R6, R9) and microalgal consortium (CP1) from aqueous medium after 12 days of incubation at 28 °C, 3000-3500 lux light intensity (Ci = 5 mg L^{-1}).

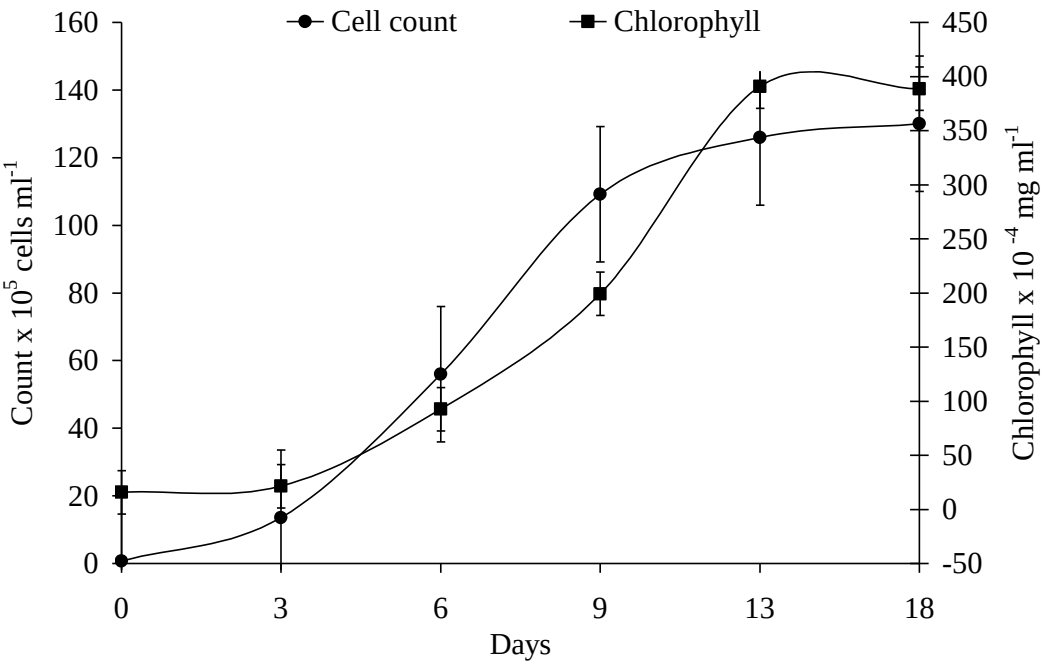


Fig. 9. Growth curve of *Chlorella* sp. (R1) as a function of Chlorophyll and Cell count.

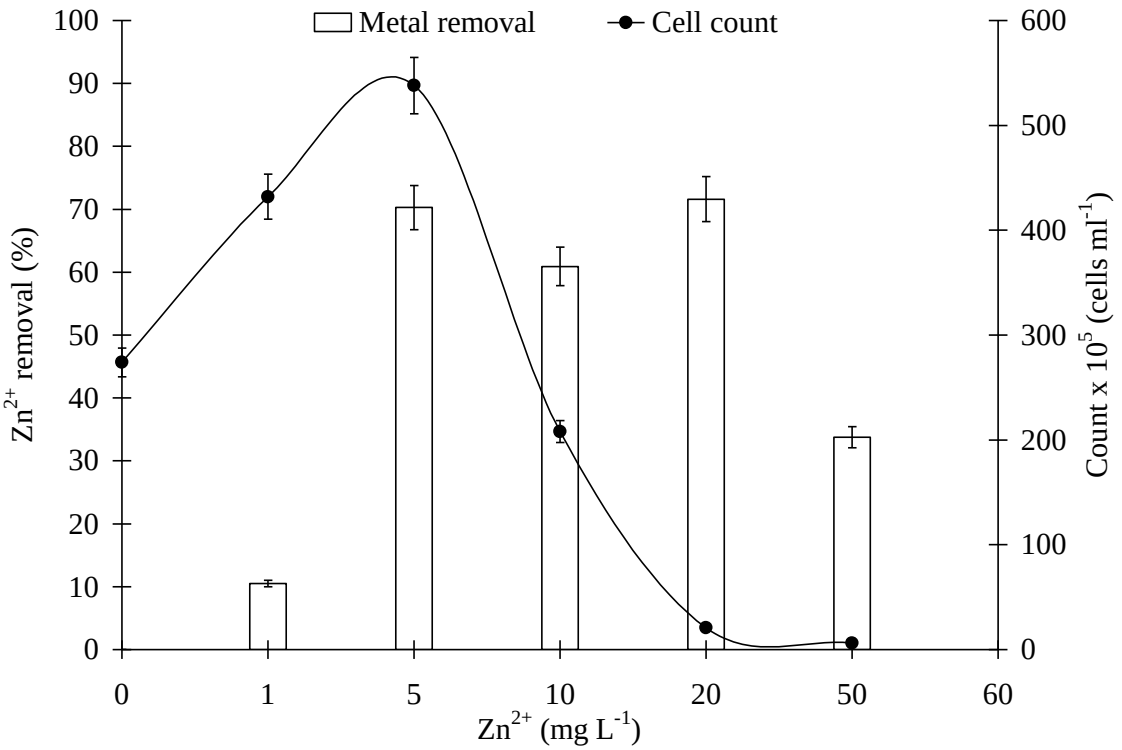


Fig. 10. Endpoint growth of *Chlorella* sp. (R1) at varying concentration of Zn^{2+} and its removal.

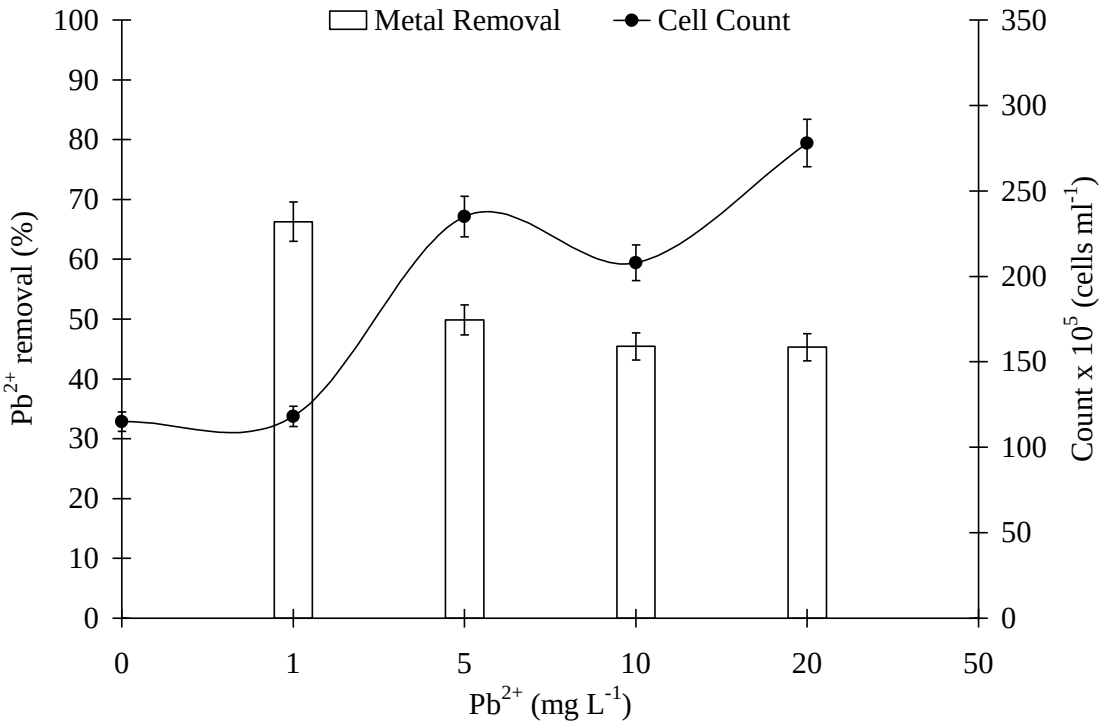


Fig. 11. Endpoint growth of *Chlorella* sp. (R1) at varying concentration of Pb^{2+} and its removal.

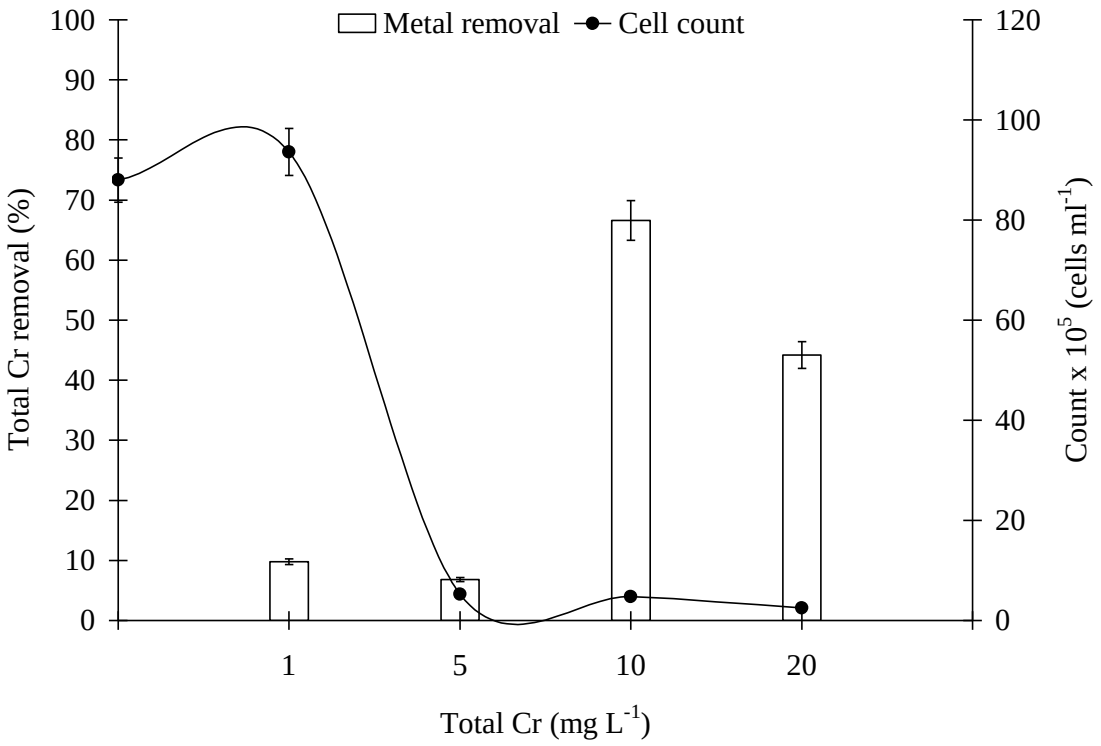


Fig. 12. Endpoint growth of *Chlorella* sp. (R1) at varying concentration of total Cr and its removal.

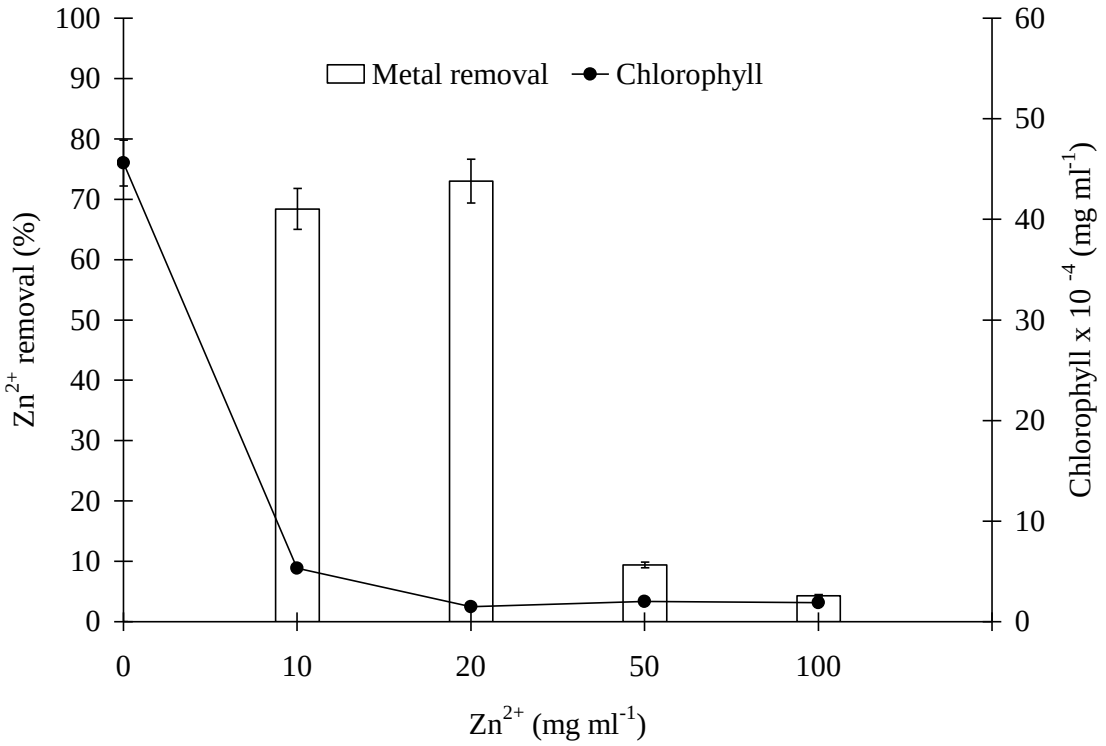


Fig. 13. Endpoint growth of algal consortium (CP1) at varying concentration of Zn^{2+} and its removal.

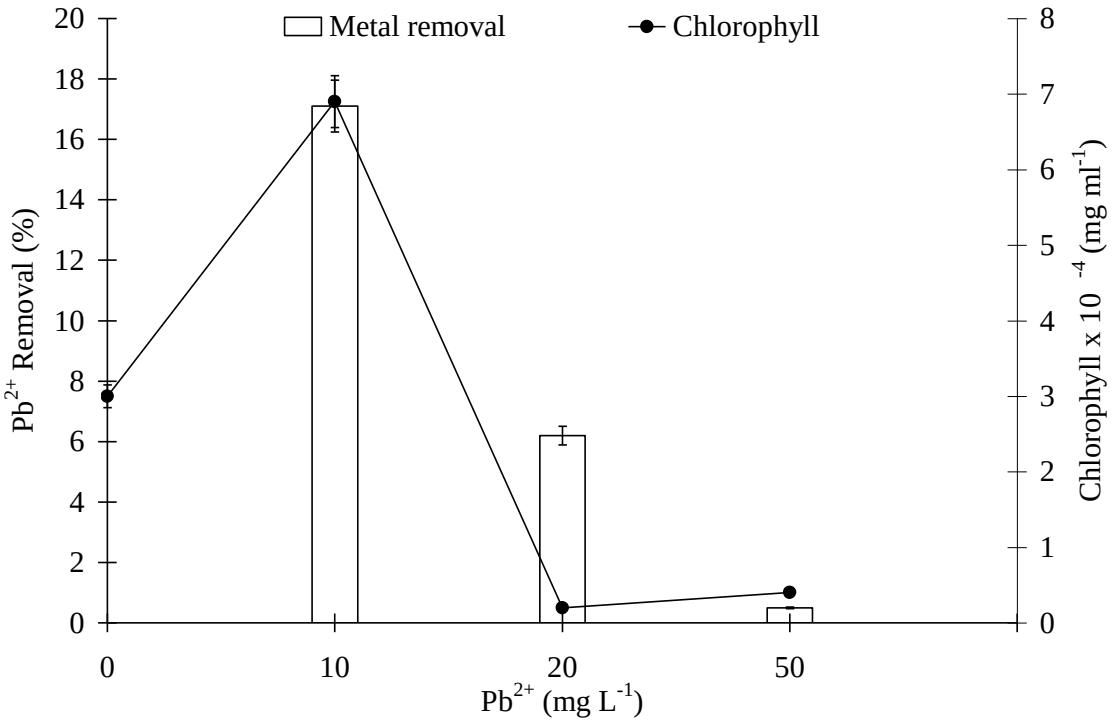


Fig. 14. Endpoint growth of algal consortium (CP1) at varying concentration of Pb^{2+} and its removal.

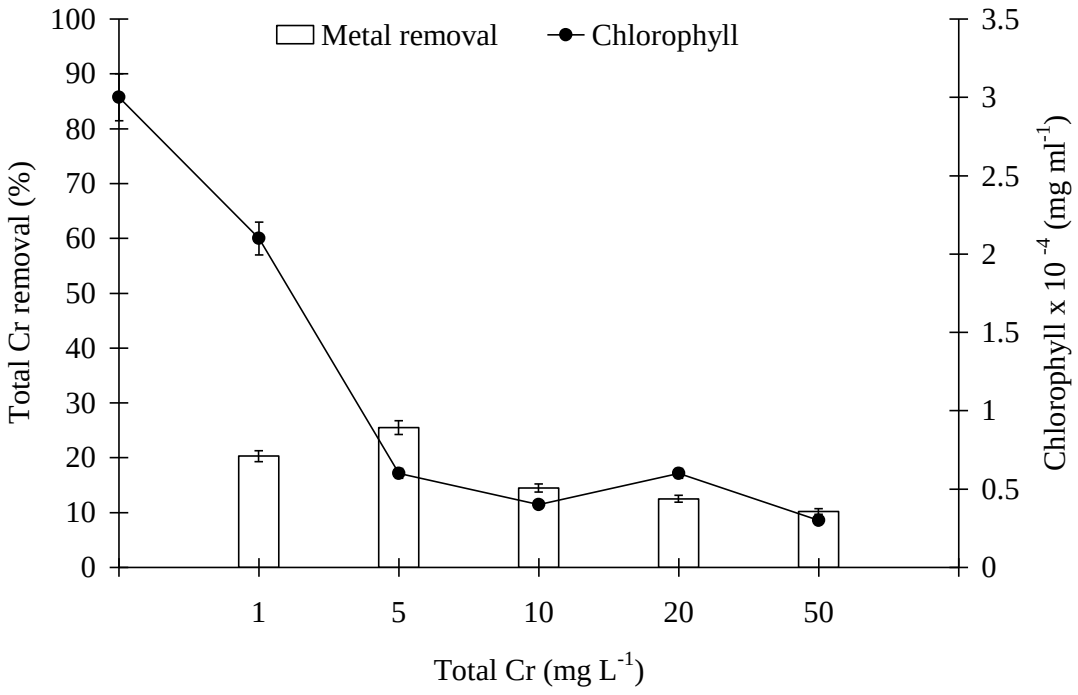
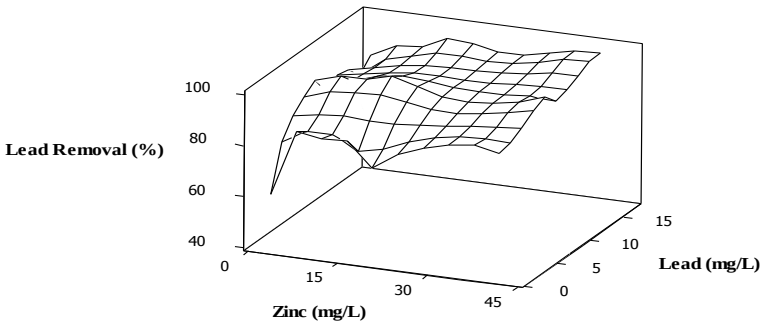
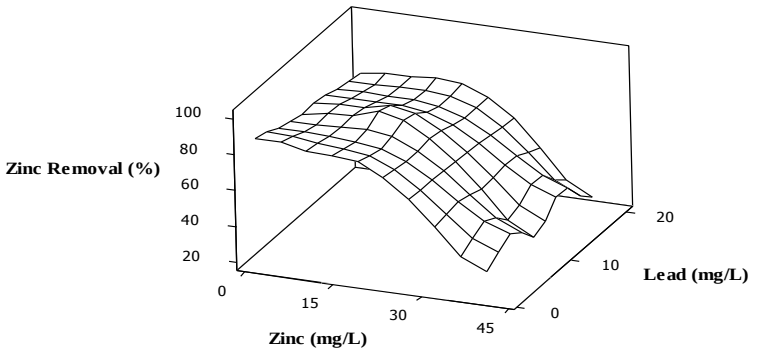


Fig. 15. Endpoint growth of algal consortium (CP1) at varying concentration of total Cr and its removal.



(A)



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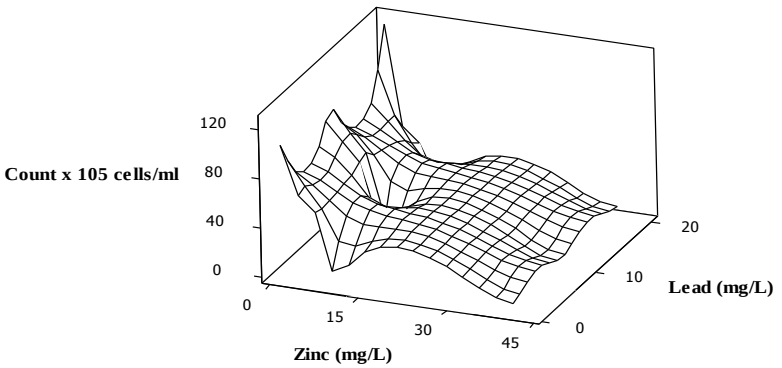
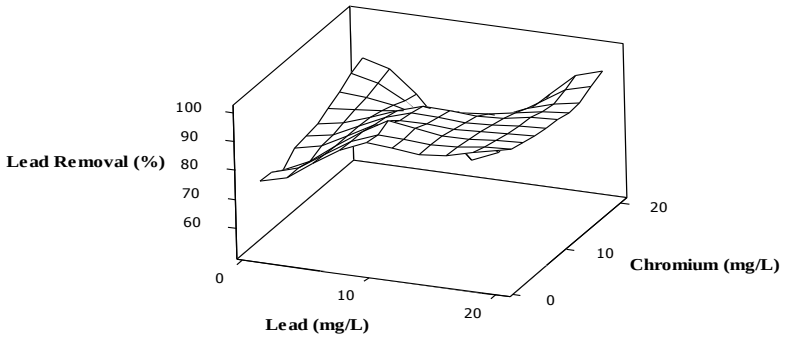
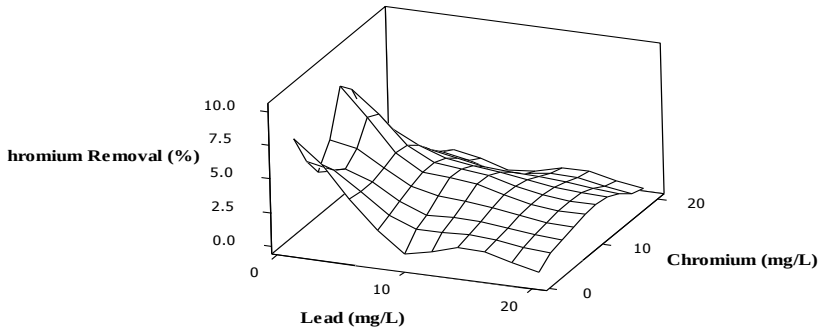


(C)

Fig. 16. Surface plots showing effect of increasing Zn^{2+} and Pb^{2+} concentration in bimetallic conditions on (A) Zn^{2+} removal (B) Pb^{2+} removal and (C) growth of *Chlorella* sp. (R1).



(A)



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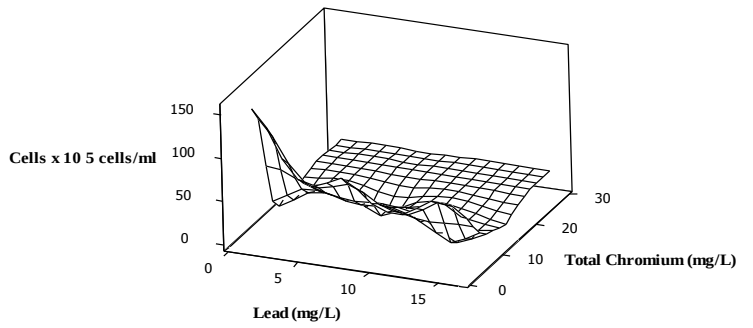
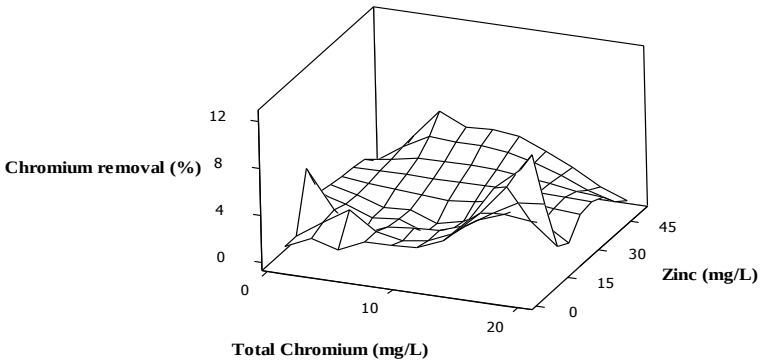
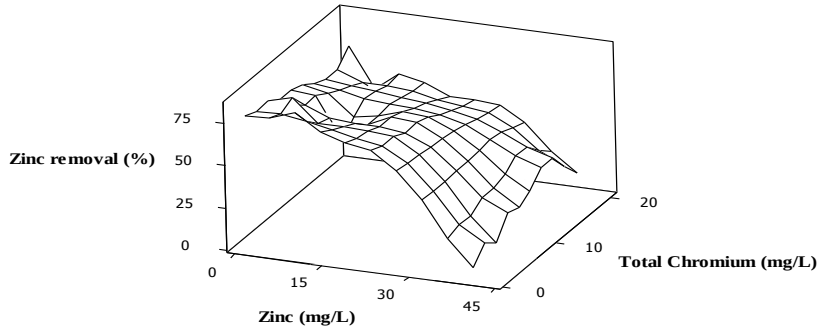


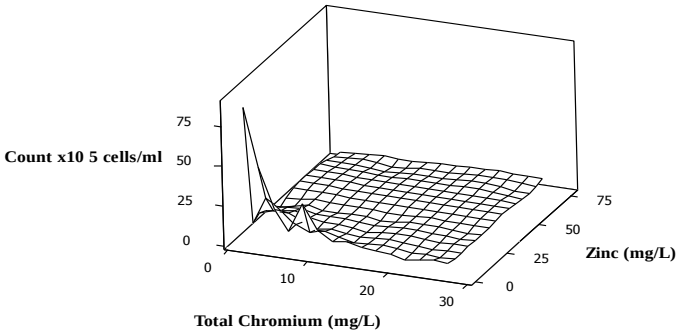
Fig. 17. Surface plots showing effect of increasing Pb^{2+} total Cr concentration in bimetallic conditions on (A) Pb^{2+} removal (B) Total Cr removal and (C) growth of *Chlorella* sp. (R1).



(A)

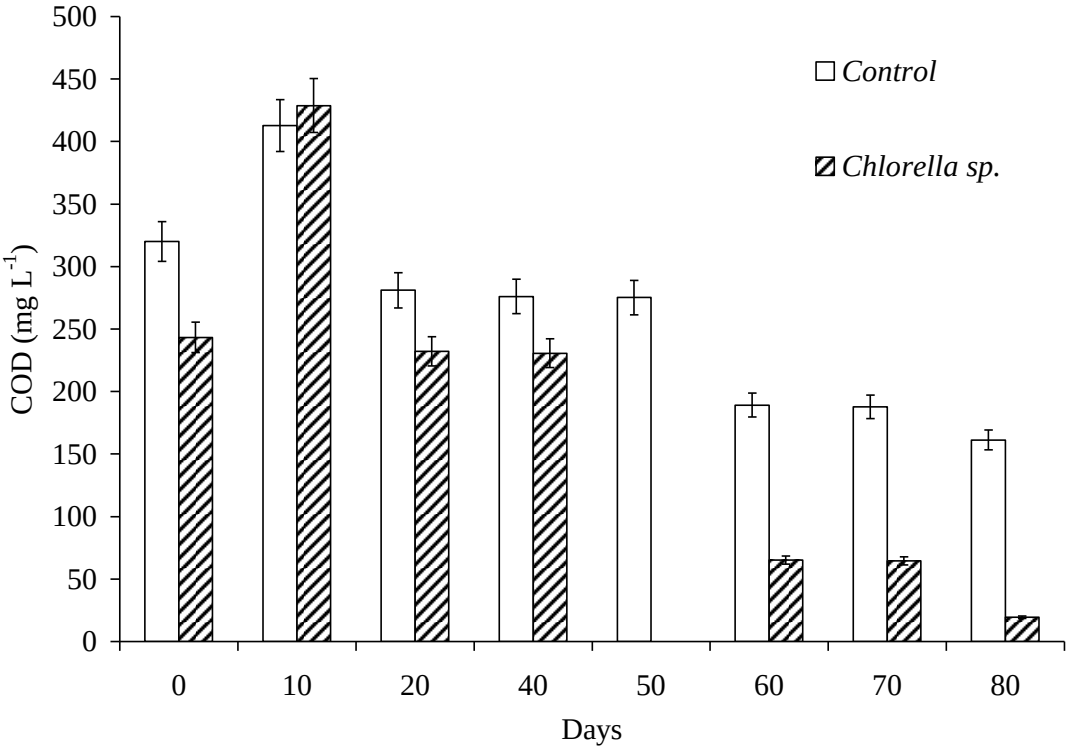


(B)

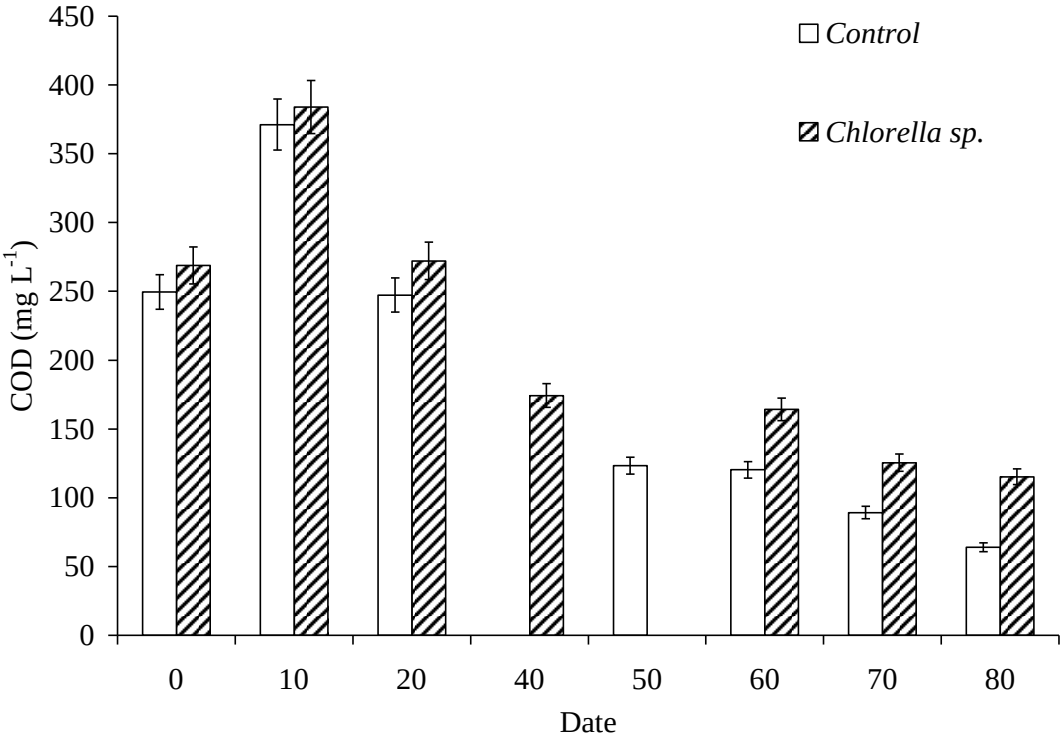


(C)

Fig. 18. Surface plots showing effect of increasing Zn^{2+} and total Cr concentration in bimetallic conditions on (A) Total Cr removal (B) Zn^{2+} removal and (C) growth of *Chlorella* sp. (R1).

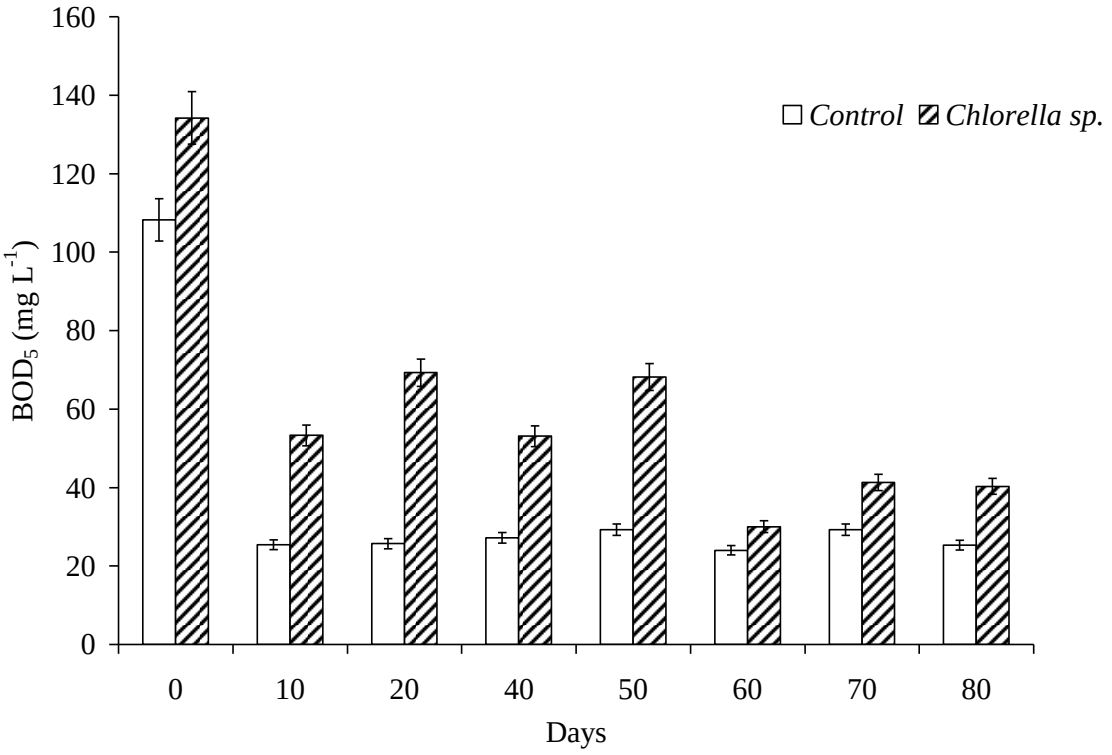


(A)

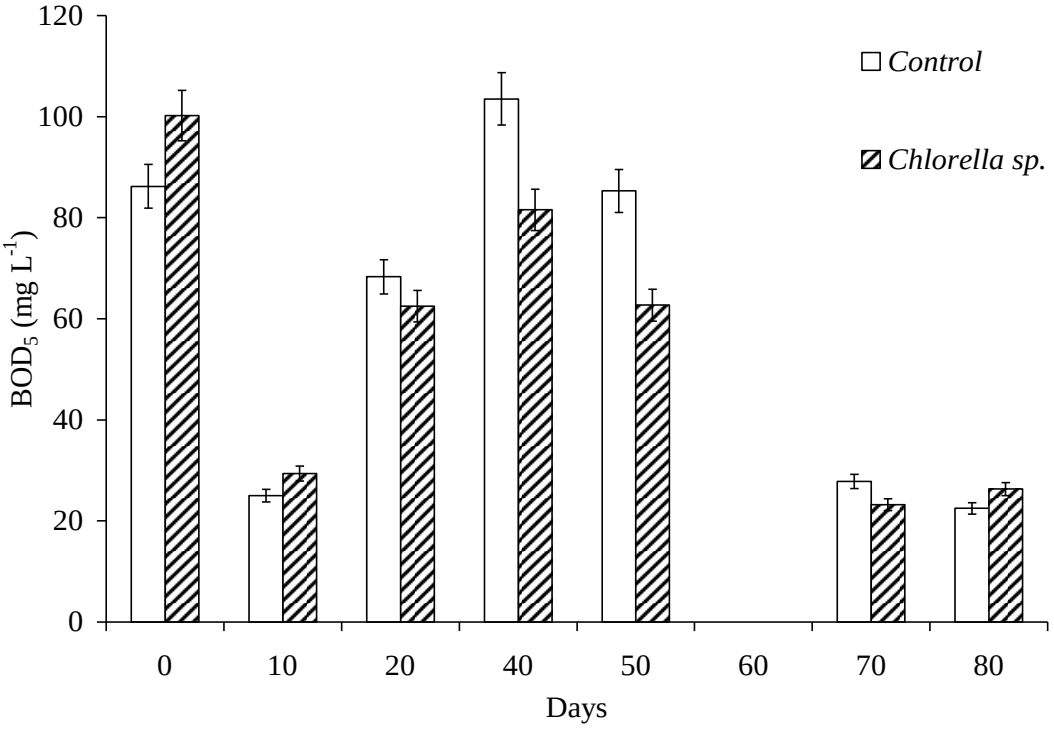


(B)

Fig. 19. Effect of *Chlorella* sp. (R1) on COD removal (A) Indoor (B) Outdoor conditions



(A)



(B)

Fig. 20. Effect of *Chlorella* sp. (R1) on BOD_5 removal (A) Indoor (B) Outdoor conditions

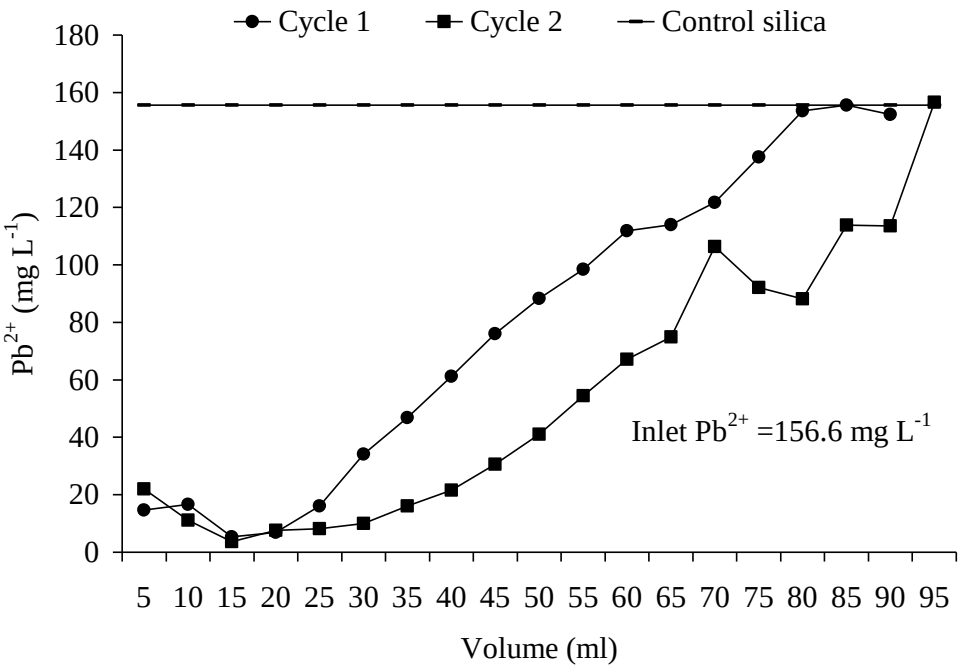


Fig. 21. Removal of Pb^{2+} in silica immobilized *Chlorella* sp. (R1) biomass (2g) in packed bed column with continuous flow of inlet aqueous solution containing 156.6 mg L^{-1} Pb^{2+}, pH 4.3.

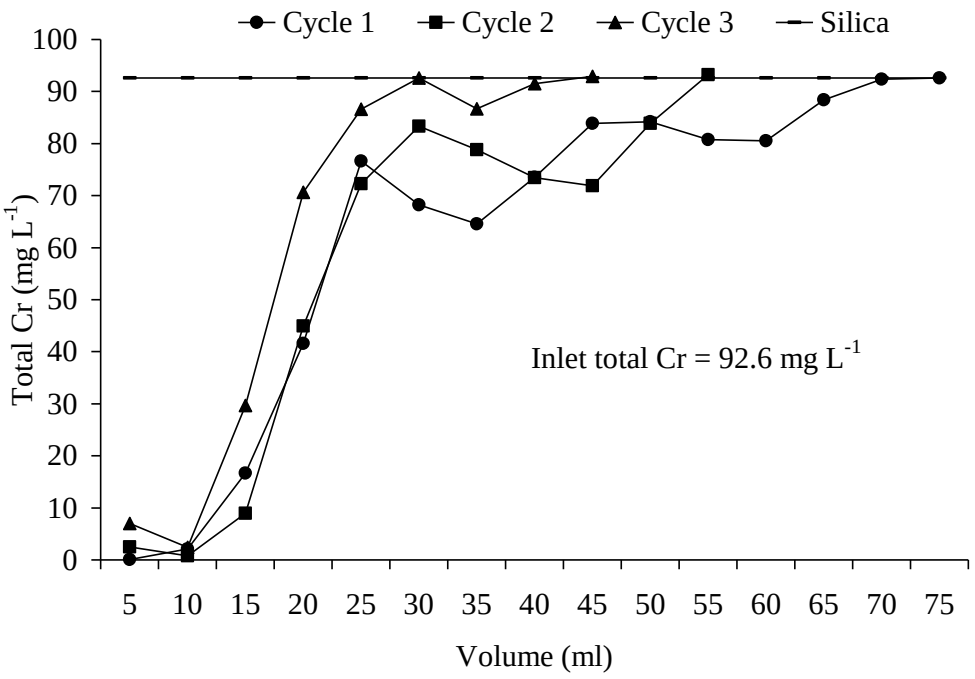


Fig. 22. Removal of total Cr in silica immobilized *Chlorella* sp. (R1) biomass (2g) in packed bed column with continuous flow of inlet aqueous solution containing 92.6 mg L^{-1} total Cr, pH 5.7.

lj

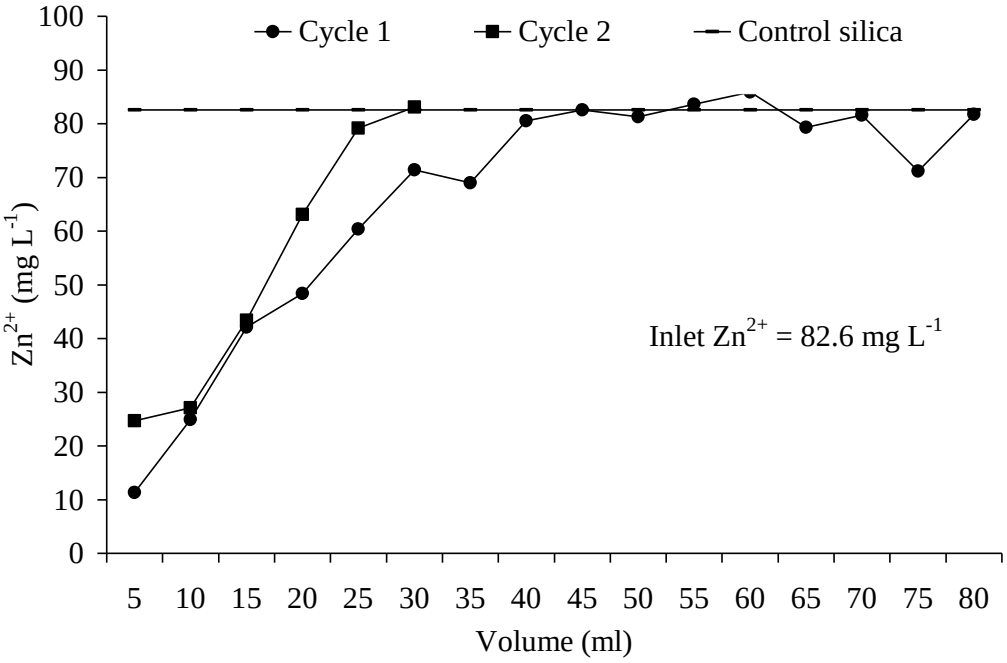


Fig. 23. Removal of Zn^{2+} in silica immobilized *Chlorella* sp. (R1) biomass (2g) in packed bed column with continuous flow of inlet aqueous solution containing 82.6 mg L^{-1} Zn^{2+}, pH 5.5.

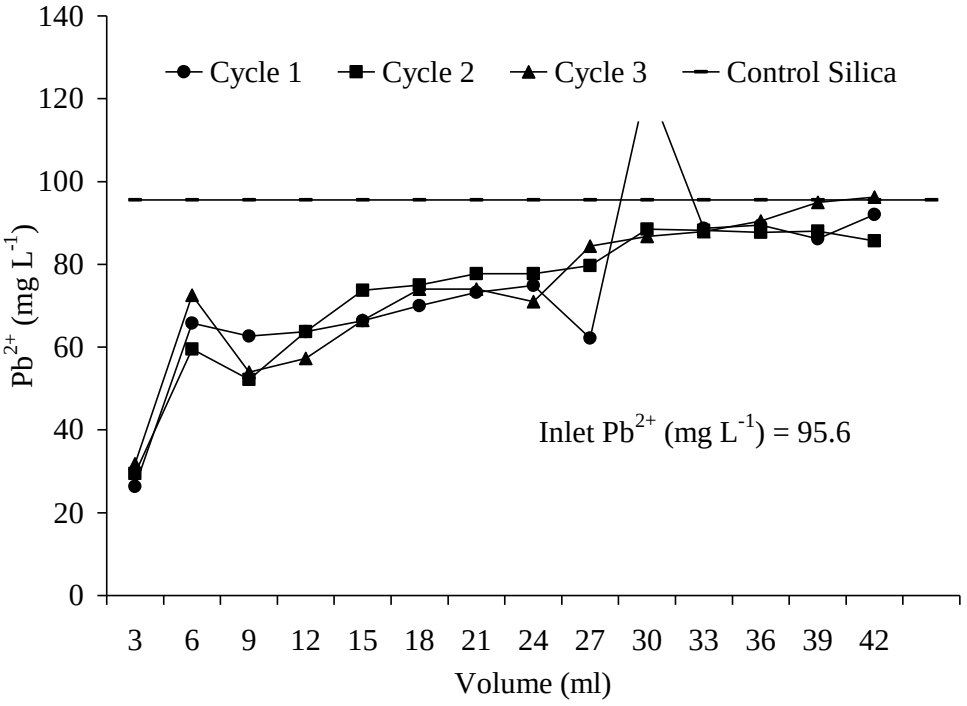


Fig. 24. Removal of Pb^{2+} in silica immobilized *Chlorella* sp. (R1) biomass (1g) in packed bed column with continuous flow of inlet aqueous solution containing 95.6 mg L^{-1} Pb^{2+}, pH 4.1.

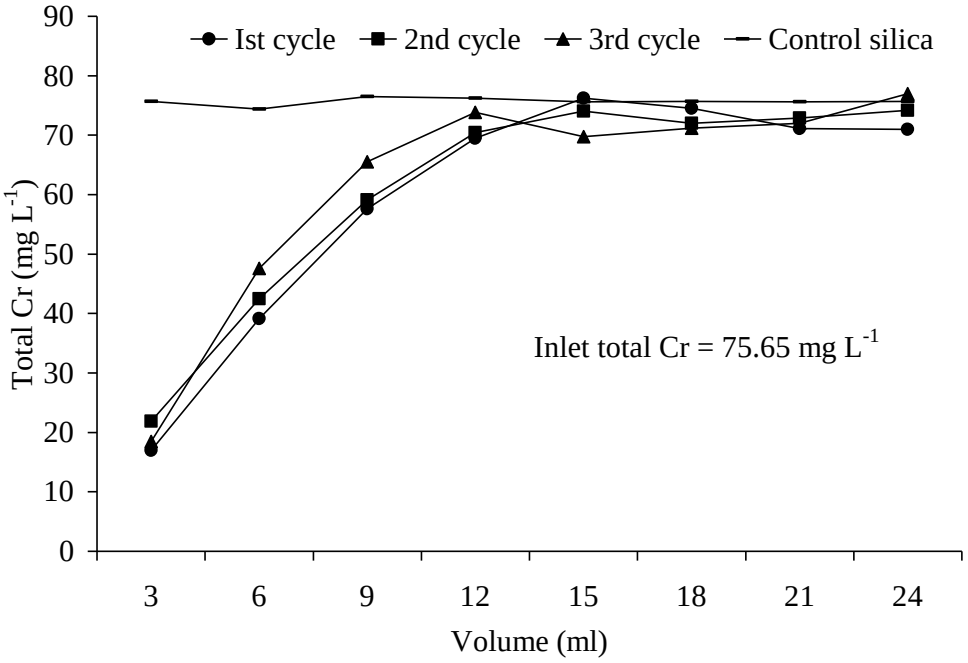


Fig. 25. Removal of total Cr in silica immobilized *Chlorella* sp. (R1) biomass (1g) in packed bed column with continuous flow of inlet aqueous solution containing 75.65 mg L^{-1} total Cr, pH 5.5.

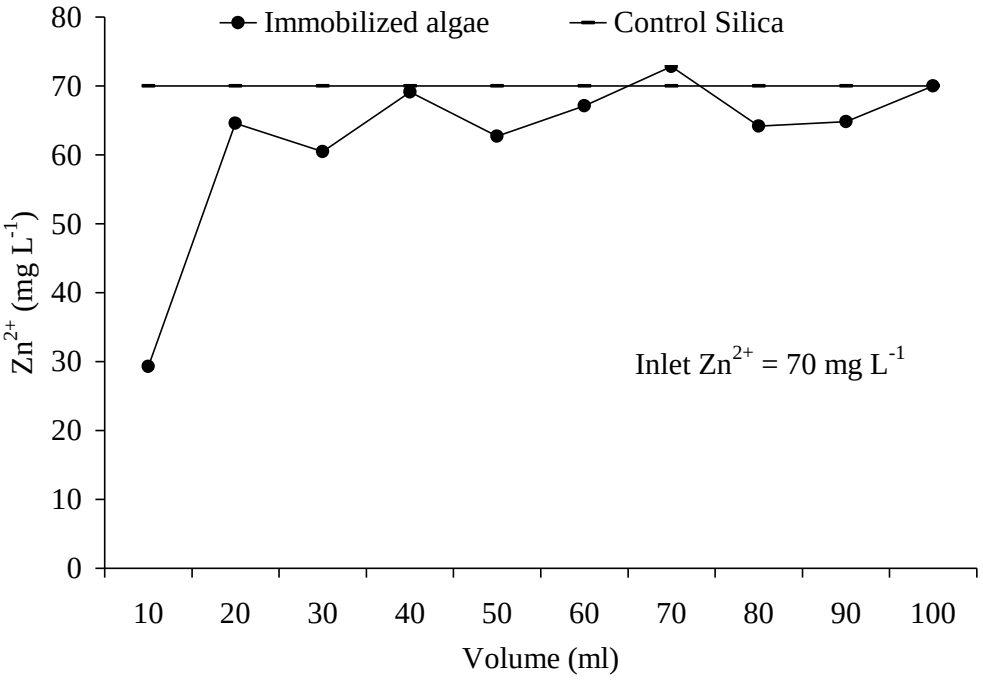


Fig. 26. Removal of Zn^{2+} in silica immobilized *Chlorella* sp. (R1) biomass (1g) in packed bed column with continuous flow of inlet aqueous solution containing 70.0 mg L^{-1} Zn^{2+}, pH 5.5.

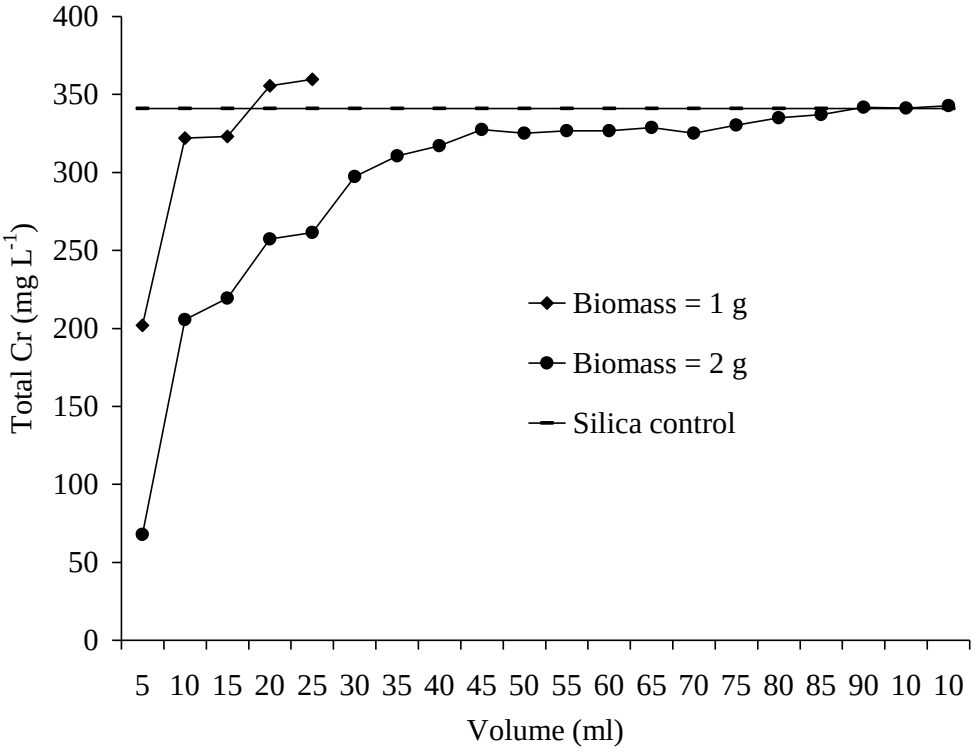


Fig. 27. Removal of total Cr in silica immobilized *Chlorella* sp. (R1) biomass (1g and 2g) in packed bed column with continuous flow of inlet industrial wastewater containing 341 mg L^{-1} total Cr, pH 1.44.

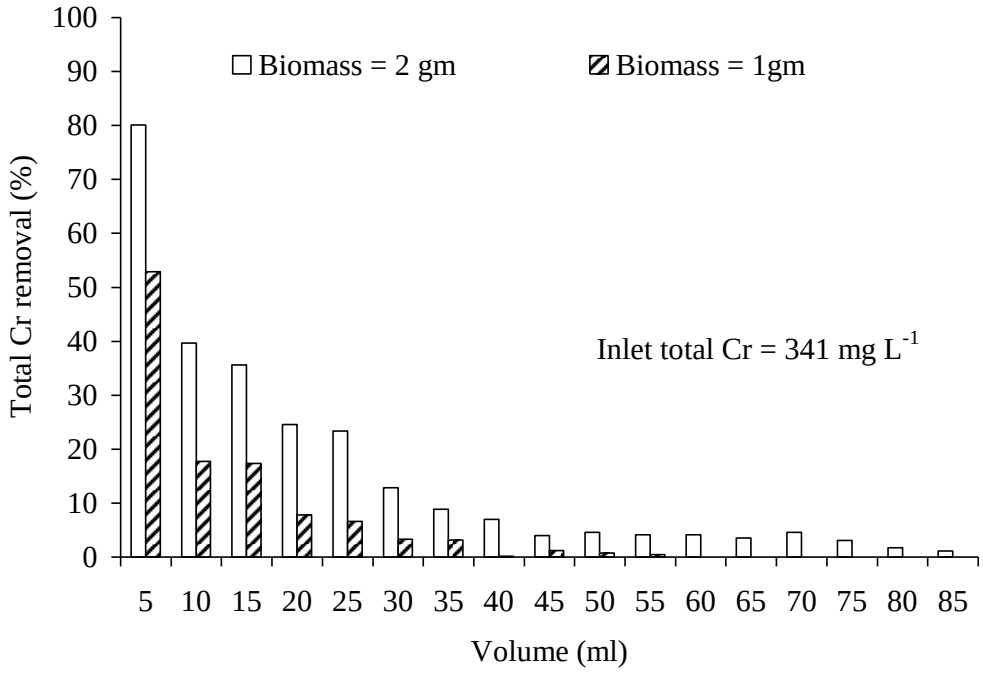


Fig. 28. Removal of total Cr in silica immobilized *Chlorella* sp. (R1) biomass (1g and 2g) in packed bed column with continuous flow of inlet industrial wastewater containing 341 mg L^{-1} total Cr, pH 1.44.

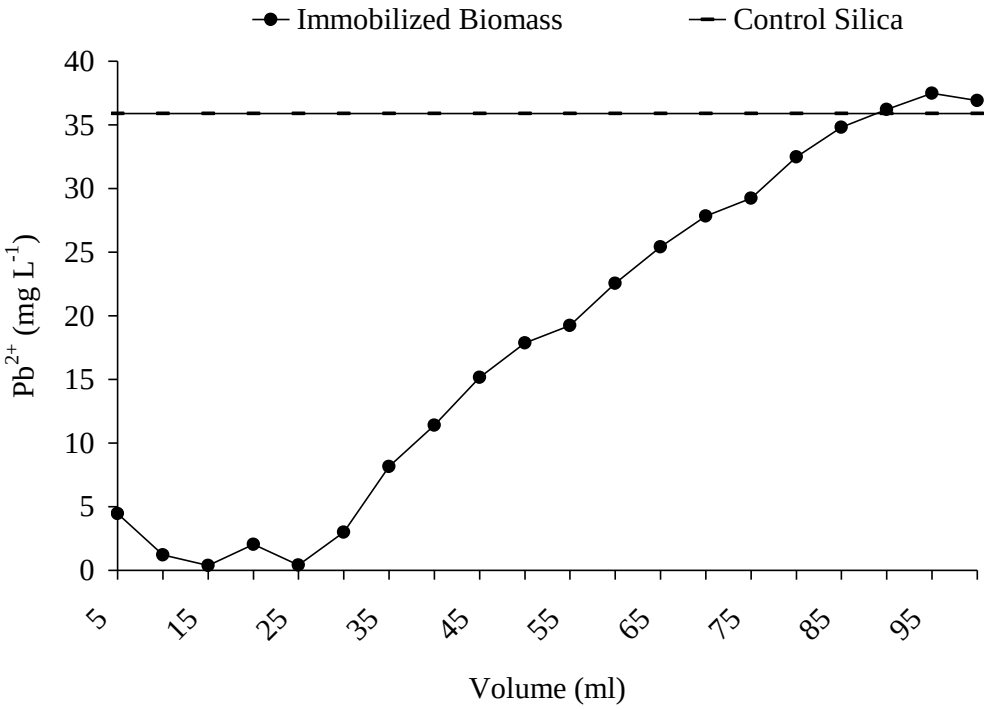
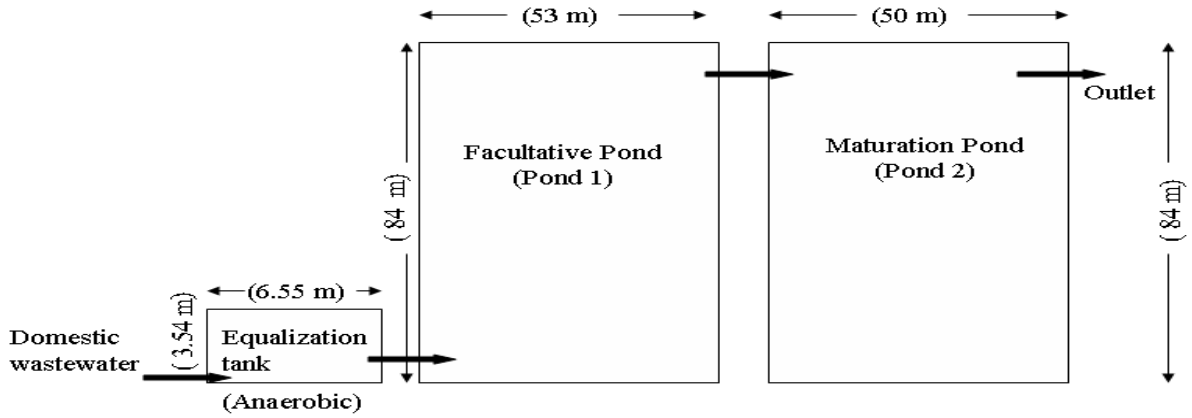


Fig. 29. Removal of Pb^{2+} in silica immobilized microalgal consortium (CP1) biomass (1.5 g) in packed bed column with continuous flow of inlet aqueous solution containing 33.90 mg L^{-1} Pb^{2+}, pH 4.3.

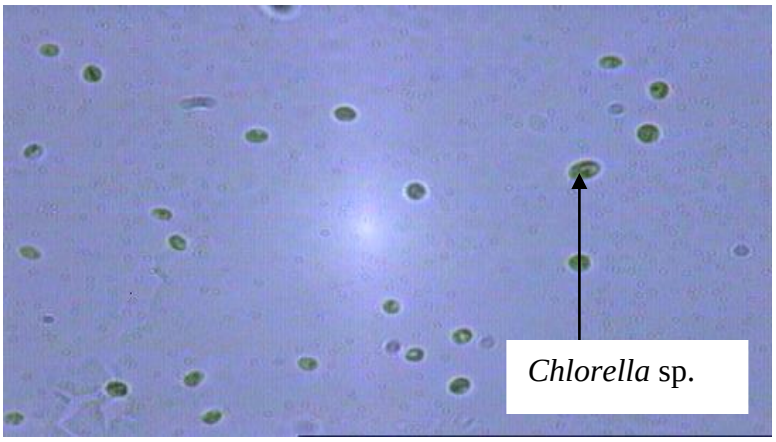
(A)

(B)

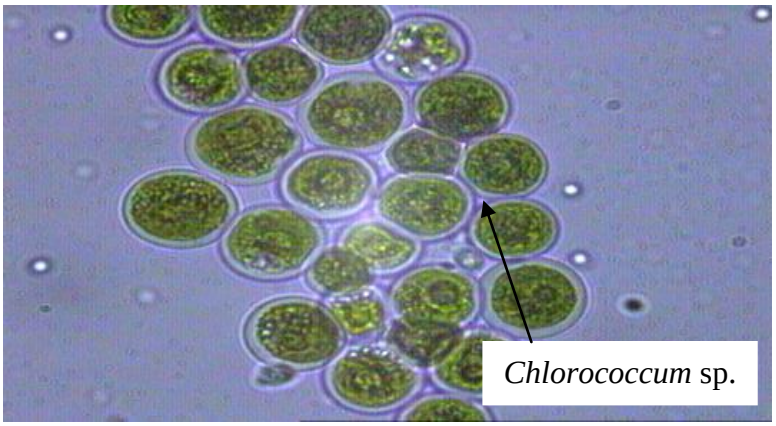


(C)

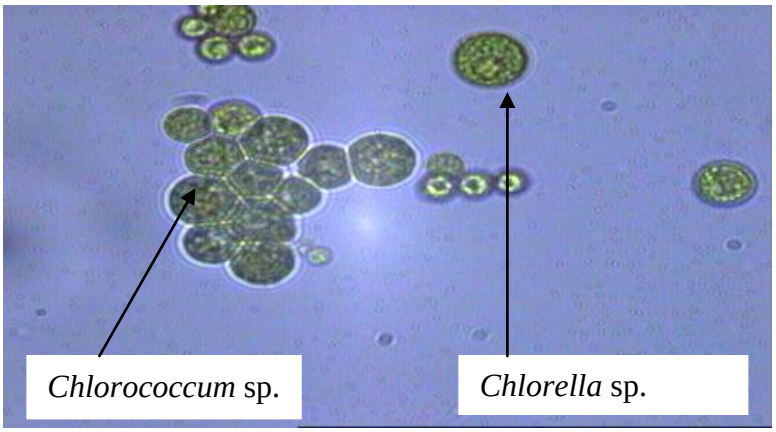
Plate 1. Wastewater stabilization pond, Sanghol village, Dist. Fatehgarh Sahib, Punjab (INDIA) (A) Facultative pond (Pond 1) (B) Maturation Pond (Pond 2) (C) Schematic diagram.


Chlorella sp.

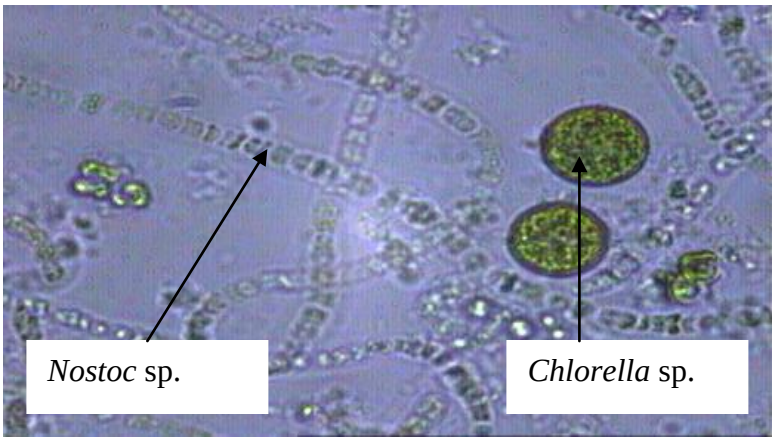
(A)


Chlorococcum sp.

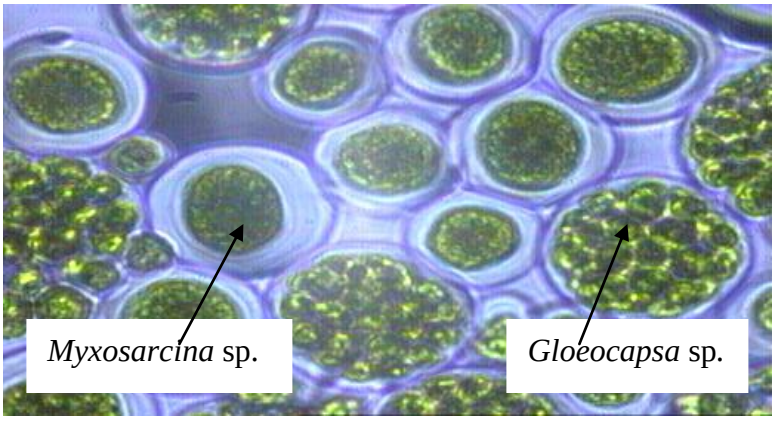
(B)


Chlorococcum sp.
Chlorella sp.

(C)



(D)



(E)

Plate 2. Photomicrographs of algal cultures (A) Algal culture R1 containing *Chlorella* sp., (B) Algal culture R2 containing *Chlorococcum* sp., (C) Algal culture R3 containing *Chlorococcum* sp., *Chlorella* sp., (D) Algal culture R5 containing *Chlorella* sp., *Nostoc* sp., (E) Algal culture R2 containing *Gloeocapsa* sp. *and Myxosarcina* sp.

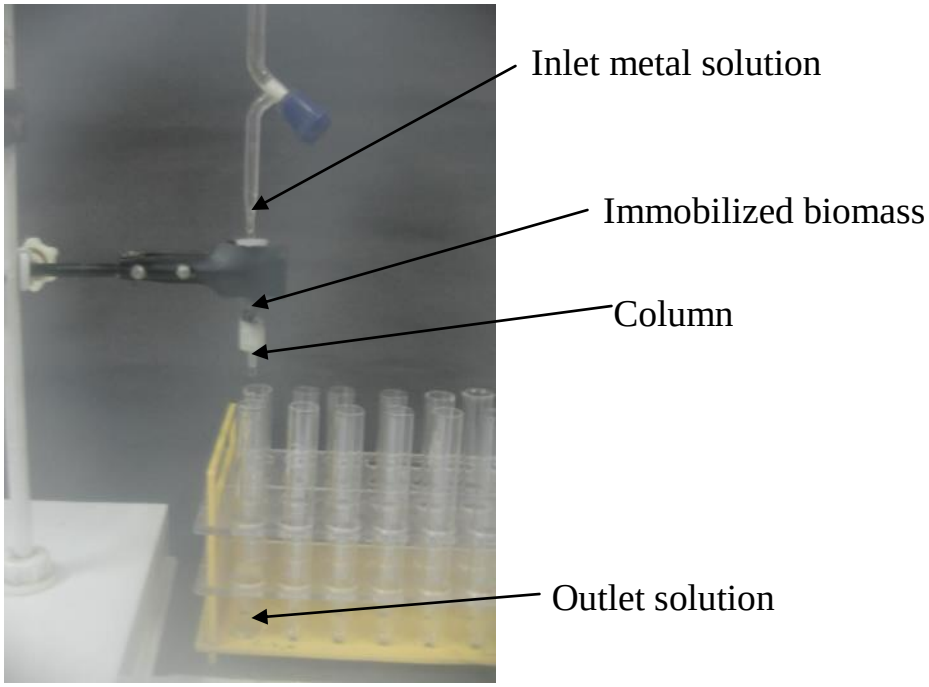
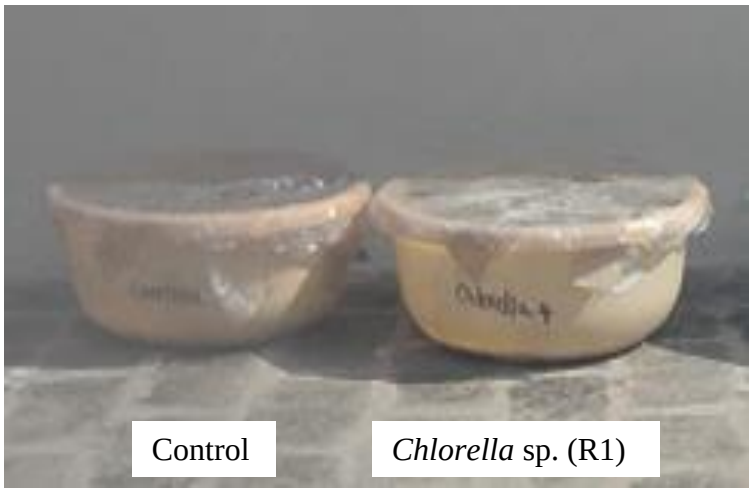


Plate 3. Experimental setup for metal removal using silica immobilized *Chlorella* sp. (R1) biomass in packed bed column.

(A)



(B)

Plate 4. Wastewater treatment in tubs (A) Indoor (B) Outdoor.