The removal of thermo-tolerant coliform bacteria by immobilized waste stabilization pond algae.

H.W.Pearson[1], A. E Marcon[2] and H.N. Melo[3]

[1]Environmental Sanitation Group, Department of Chemistry, State University of Paraiba, Av das Baraúnas, 351, Bodocongó 58109-753 Campina Grande, PB, Brazil.
[2]Dept of Biology, Federal University of Rio Grande do Norte, Natal, RN. Brazil
[3]Dept. of Chemical Engineering, Federal University of Rio Grande do Norte, Natal, RN. Brasil.
[1]howard_william@uol.com.br, [2]endresbio@yahoo.com.br, [3]henio@eq.ufrn.edu.br.

Abstract: This study investigated the potential of laboratory- scale columns of immobilized micro-algae to disinfect effluents using thermo-tolerant coliforms (TTC) as a model system. Cells of a Chlorella species isolated from a waste stabilization pond complex in Northeast Brazil were immobilized in calcium alginate, packed into glass columns and incubated in contact with TTC suspensions for up to 24 hours. Five to six log removals of TTC were achieved in 6 hours and 11 log removals in 12 hours contact time. The results were similar under artificial light and shaded sunlight. However little or no TTC removal occurred in the light in columns of alginate beads without immobilized algae present or when the immobilized algae were incubated in the dark suggesting that the presence of both algae and light were necessary for TTC decay. There was a positive correlation between K_b values for TTC and increasing pH in the effluent from the immobilized algal columns within the range pH 7.2 and 8.9. The potential of immobilized algal technology for wastewater disinfection may warrant further investigation.
Keywords: immobilized Chlorella; Thermo-tolerant coliform removal; waste stabilization ponds; alginate.

INTRODUCTION

The use of immobilized micro-algal systems for the industrial production of metabolites, the removal of nutrients, metals and organic pollutants from aqueous systems, measurements of toxicity and algal hydrogen production have been recently reviewed by Morreno-Garrido (2008). Applications of immobilized algal systems to wastewater treatment have tended to concentrate on nutrient and metal removal and co-immobilized systems of algae and bacteria have also been studied (Megharaj et al., 1992; Hoffman, 1998; Tam et al., 2000; Bashan et al., 2002).

The use of waste stabilization ponds and high rate pond systems to treat domestic and industrial wastewaters is well established. The advantage of these systems over electro-mechanical processes in terms of sewage treatment has been their ability to produce effluents of good microbiological quality suitable for effluent re-use without the need for additional disinfection. Pond disinfection processes and the role of micro algae have been extensively and recently reviewed by Davies-Colley (2005). However, little attention has been paid to the potential use of immobilized algal systems for effluent disinfection.

In the present study the use of immobilized pond algae to disinfect wastewater effluents is investigated using thermo-tolerant coliforms (TTC) as a bacterial model. An attempt is also made to determine the factors affecting the bacterial die-off process by a species of Chlorella (originally isolated from a tropical maturation pond), when immobilized in calcium alginate beads.

MATERIAL AND METHODS

Isolation and maintenance of microorganisms:

The green algal species Chlorella and the thermo-tolerant coliform cultures (TTC) used in this study were originally isolated from the final maturation pond effluent of the Ponta Negra waste stabilization pond complex of the city of Natal, Rio Grande do Norte, Northeast Brazil.

Uni-algal pond isolates of Chlorella were cultured aseptically in 2L Erlenmeyer flasks containing 500ml of Bold's media (Borowitzka, 1988). The cultures were grown under continuous white fluorescent light (40W) giving a light intensity of approximately $85\mu m.s^{-1}.m^{-2}$. The ambient temperature was $28 \pm 3^{o}C$ and the flasks were shaken manually every 8h. The algal cells were harvested by centrifugation (3000g for 10min) after 15 days growth for subsequent immobilization in alginate beads.

The thermo-tolerant coliforms (TTC) colonies were isolated from the same maturation pond effluent using the membrane method (APHA 1998). Subsequently a TTC inoculum incorporating several membrane colonies was grown in TTC liquid medium (Difco) at $44^{o}C$ for 24h. One ml of the concentrated broth culture was diluted in 100ml of sterile distilled water (pH adjusted to 7.0) to give a final TTC concentration of approximately 10^{11} cells $100ml^{-1}$. This suspension of TTC was used to study TTC die-off in the immobilized Chlorella columns. TTC samples taken from the immobilized algae and control columns were analyzed using the same membrane method.

Algal immobilization in alginate:

The washed centrifuged pellet of cultured Chlorella cells was re-suspended in sterile distilled water (pH 7.0) to give a cell concentration of approximately $7x10^{7}$ cells ml^{-1}. The algal suspension was mixed 1:1 with 4% autoclaved sodium alginate solution and mixed using a rotary mixer. The final 2% mixture of sodium alginate containing the Chlorella cells was placed in a burette and added drop-wise into a stirred solution of 0.4M $CaCl_2$ producing beads of immobilized Chlorella in a matrix of calcium alginate. Bead size was approximately 4mm in diameter. Each bead contained approximately 10^{6} algal cells and the chlorophyll *a* concentration was approximately $3.6\mu g$ chl *a* $bead^{-1}$ as estimated by methanol extraction (APHA 1998). The alginate control beads without Chlorella were prepared similarly except the 4% sodium alginate solution was initially mixed with distilled water (Megharaj *et al*., 1992; Tam and Wong, 2000).

Bioreactors

The columns of immobilized algae were prepared by filling 100ml burettes with the immobilized algal beads or control beads suspended in distilled water to prevent the formation of air bubbles between the beads. Immediately prior to experimentation the columns were eluted with the TTC suspension to replace the distilled water. These simple reactors were operated under batch conditions and samples of the TTC suspension removed at different time intervals.

RESULTS AND DISCUSSION

The glass column containing beads of immobilized Chlorella cells originally isolated from a waste stabilization pond and the control column filled with alginate beads but without algae were eluted with a mixed culture of thermo-tolerant coliforms (TTC) re-suspended in ringers solution to displace any algal culture medium before finally filling and leaving to incubate in fluorescent light (approx. 85 µmols $s^{-1}.m^{-2}$) for 24h. The ambient air and column effluent temperatures varied between 27.5 and 30.5°C and 28-29.5°C respectively. Samples of column effluents were removed after 0.05h, 3h and thereafter every three hours and analyzed for TTC. The mean results for six such experiments in which the age of the immobilized algal beads varied between 10 and 40 days are presented in Figure 1.

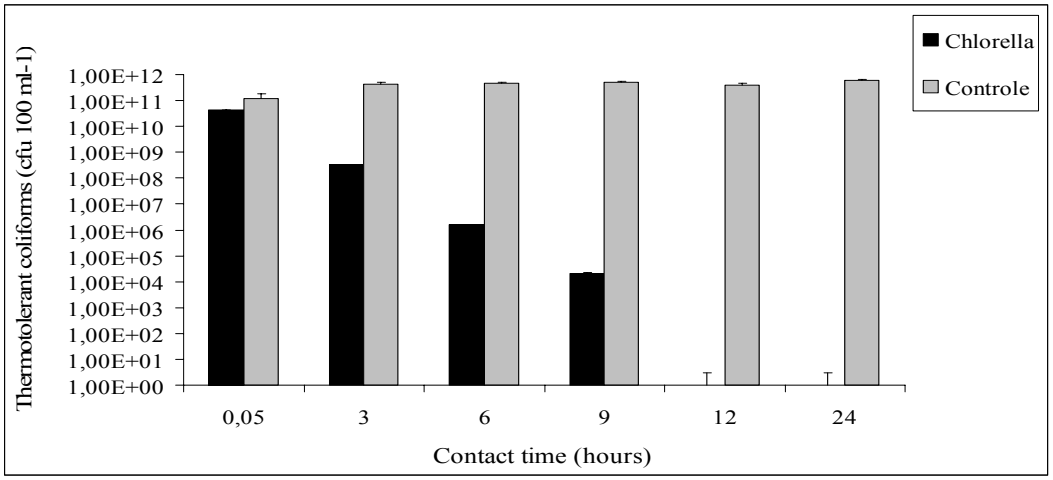

Figure 1. Thermo tolerant coliforms (TTC) removal with time by columns of immobilized Chlorella cells incubated in artificial light. The controls were columns of alginate beads without immobilized algae. The values are the means of triplicates from six experiments. The maximum standard deviation values (⊤), for $p \leq 0.05$.

Removal of TTC only occurred in the columns with immobilized Chlorella cells and in comparison with TTC removal rates in maturation ponds the process was rapid with a 5 to 6 log removal of TTC in 6h and complete removal (<11 logs) in less than 12h (Curtis et al 1992). When the same experiments were repeated with immobilized algal beads that had been prepared 63 to 72 days previously the results were similar than those with the younger algal beads (Figure 2). This suggests that these immobilized algal systems have a prolonged active life span in terms of TTC removal. Indeed Chen (2001) demonstrated that stored immobilised Scenedesmus cells immobilized in alginate remained viable for over two years and Megharaj et al (1992) demonstrated algal growth within the alginate beads.

In another series of experiments the immobilized columns of Chlorella were exposed to shaded natural light with the intensity varying between 604 and 906 µmols.m^{-2} s^{-1}. However in this case the control columns also contained immobilized algal beads but the

columns were incubated in the dark by enclosing them in aluminium foil. The maximum exposure period was six hours so as to utilise a period of relatively even light intensity between 10.00 and 16.00 (intensity) and the results are presented in Figure 3. The mean ambient and effluent temperatures of 27 to 34.5°C and 28.5 to 30°C respectively were higher than in the experiments under artificial light but statistically there was no difference in the rates of TTC die-off between columns incubated in shaded sunlight and

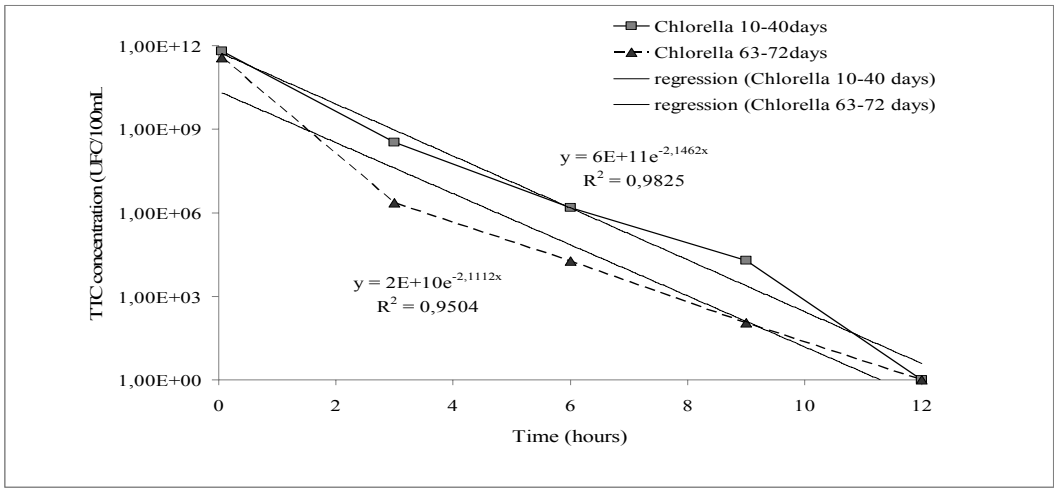

Figure 2. Thermo-tolerant coliforms (TTC) removal by columns of Chlorella cells immobilized for 10 to 40 days and 63 to 72 days when incubated under artificial illumination. Values are the means of triplicates from 6 experiments for each age group of the algal beads.

the previous studies using low intensity fluorescent light. Nevertheless algae together with light were necessary for efficient TTC die-off since little die-off occurred in the immobilized algal columns incubated in the dark (Figure 3).

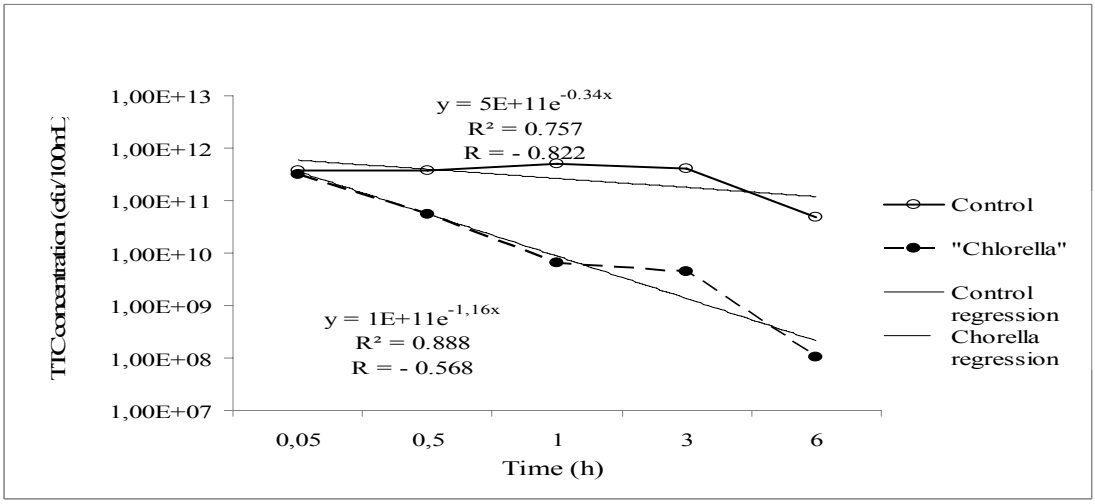

Figure 3. TTC removal by columns of immobilized Chlorella cells under natural light conditions. The controls were columns of immobilized algae kept in the dark by encapsulation in aluminium foil to exclude light.

The lack of apparent TTC decay in the light controls comprising alginate beads without immobilized algae in the artificial light experiments and with TTC suspensions in Ringers solution incubated under shaded sunlight conditions for 12 hours could perhaps be due to the relatively low light intensities used in these experiments.

The pH values of effluent samples from the immobilized Chlorella columns measured at the time of TTC sampling are presented in Table 1 which shows the combined mean results of eight experiments with artificial light and six experiments with shaded sunlight (6h incubation). The pH values for the two types of controls i.e., light without algae and dark with algae were also combined. There was a steady increase in effluent pH with time of light exposure in the immobilized algal columns reaching a value of pH 8.3 after 6 hours and pH 8.9 within 12 hours. In contrast the pH varied little in the light and dark controls over the same period. This might be expected since an increase in pH can be attributed to the photosynthetic activity of the algae immobilized in the alginate beads.

The polysaccharide matrix of the alginate beads is known to be permeable to liquids and gases (Moreno-Garrido; 2008). Samples of immobilized algal beads suspended in culture medium also demonstrated oxygen evolution in the light when placed in the well of a Clarke-type oxygen electrode (data not shown).

Table 1. pH values (±SE) in the effluents of immobilized Chlorella columns at the time of TTC sampling. The results for both light regimes have been combined. The control values are a combination of results for columns of alginate beads without algae incubated in the light and columns of immobilized algae incubated in the dark.

Contact time (h)	Chlorella	Control
0,05	7.23 (±0.1)	7.35 (±0.2)
0,5	7.25 (±0.5)	7.20 (±0.3)
1	7.73 (±0.3)	7.15 (±0,2)
3	8.35 (±0.2)	7.41 (±0.3)
6	8.33 (±0.1)	7.22 (±0.2)
9	8.59 (±0.4)	7.57 (±0.1)
12	8.90(±0.3)	7.75 (±0.4)

There is considerable controversy and differences of opinion as to the hydraulic flow regimes predominating in ponds of different geometries and of the impact of pond geometry on pond performance (Pearson et al., 1995; Shilton and Sweeney, 2005). In this study the values for the first order rate constant for TTC removal (K_b d^{-1}), were calculated assuming plug flow in the algal columns using the equation of von Sperling (1999) when comparing the performance of Brazilian maturation ponds. When these were plotted against pH there was a positive linear correlation between Kb and increasing pH as shown in figure 4. It would seem that the key factors controlling the rate of TTC in maturation ponds namely elevated pH and light linked to algal photosynthesis are relevant in the immobilized algal system but the system is clearly more efficient.

Studies with larger, pilot-scale, immobilized algal columns to investigate the removal of other bacterial groups, protozoa and viruses could provide information as to the suitability of such a relatively inexpensive disinfection technology as an alternative to conventional maturation ponds since, at least in the case of the TTC model system, the disinfection time can be reduced from several days to hours.

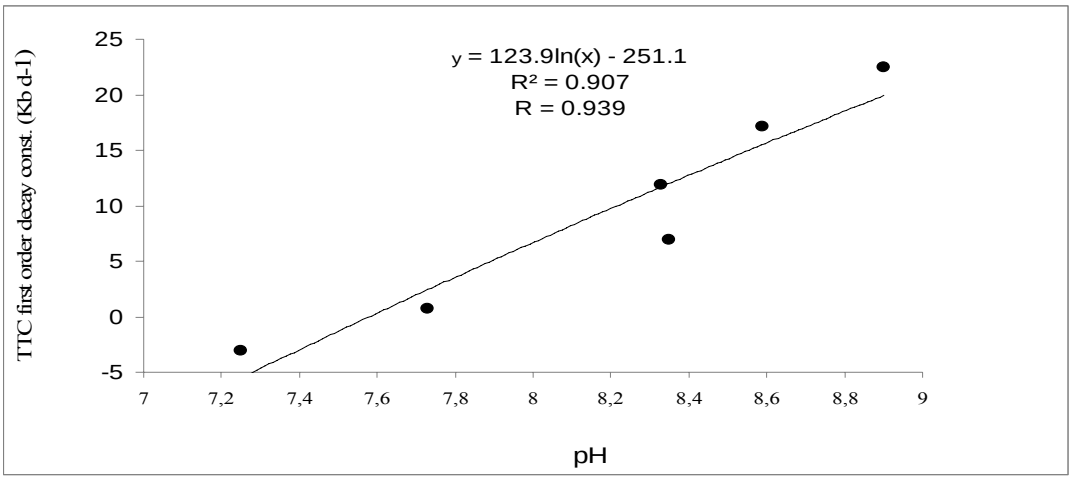

Figure 4. The relationship between TTC first order decay constants (K_b d^{-1}) and pH in effluent samples from the immobilized algal columns incubated in artificial light. The values are the means of triplicates analyses from six experiments.

Cellular immobilization technology is easy to apply and in the case of algae they can be isolated, cultured and immobilized on-site at any waste stabilization pond system or cultures obtained from algal culture collections. However, it is interesting to note that Jiménez-Pérez et al. (2004) found that N and P removal rates by immobilized green microalgae isolated from piggery manure were markedly higher than when commercially available algal isolates were used. They suggested that it was reasonable to hypothesize that species that naturally develop in wastewater, which are well adapted to the temperature and chemical changes in the medium, would show higher efficiencies than commercially available ones. The "spent" immobilized algal beads could be used as a slow release "green fertilizer" as part of an effluent reuse strategy in agriculture.

In on going studies we are investigating the efficiency of other common waste stabilization pond algae and cyanobacteria in continuous flow columns in pilot scale (2 m high and 2.5 cm in diameter), for disinfection. These studies also consider virus, protozoa and helminth egg removal and look at the efficacy of combining pathogen removal with nutrient removal from various secondary treated effluents.

CONCLUSIONS

The results of this study showed:

1. Columns of Chlorella cells immobilized in alginate beads removed 5 log concentrations of thermo-tolerant coliform bacteria in 6h and 11 logs in 12h.

2. Algal beads that had been immobilized for 72 days continued to function efficiently in non-sterile conditions.
3. The rate of TTC removal appeared to be the same when the immobilized algal columns were incubated in artificial light as in shaded sunlight conditions.
4. Apparently both light and the presence of algae were necessary for TTC removal since very little die-off occurred in the control columns containing alginate beads without immobilized algae present when incubated in the light and when the columns containing immobilized algae were incubated in the dark..
5. There was a positive correlation between the first order rate constant for TTC die-off (K_b d^{-1}) and increasing in pH in the effluents from the immobilized algal columns in the light.
6. Immobilized algal technology using algal isolates from waste stabilization ponds warrants further investigation at pilot scale, possibly as a combined disinfection and nutrient removal technology for secondary wastewater effluents. The system could operate continuously combining artificial light periods with sunlight so as to operate continuously over a 24h cycle.

ACKNOWLEDGEMENTS

The authors wish to thank FINEP/PROSAB for financial support for this research in terms of capital and consumable costs and CNPq for a scholarship to HWP. This work is dedicated to the memory of Professor Salomão Anselmo Silva who died recently and who made a significant contribution to waste stabilization research over the last 30 years.

REFERENCES

APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition.American Public Health Association, American Water Works Association and WaterPollution Control Federation, Washington, DC.

Bashan, L. E., Moreno, M., Hernandez, J. P. and Bashan, Y. (2002). Removal of ammonium and phosphorus ions from synthetic wastewater by the micro-algae *Chlorella vulgaris* co-immobilized in alginate beads with the micro-algae growth-promoting bacterium Azospirillum brasilense. *Water Research*, **36**, 2941-2948.

Borowitzka, M. A. (1988). *Micro-algal Biotechnology* Cambridge University Press. Cambridge.

Chen, Y. (2001). Immobilized microalga *Scenedesmus quadricauda* (Chlorophyta, Chlorococcales) for long-term storage and for application in fish culture water quality control. *Aquaculture*. **195**(1-2), 71-80.

Curtis, T.P., Mara, D.D. and Silva, A.S. (1992). The effect of sunlight on faecal coliforms in ponds – implication for research and design. *Water Science and Technology*, **26**(7-8), 1729-1738.

Davies-Colley, R. (2005). Pond disinfection. *In Pond Treatment Technology*, Shilton (edit). IWA publishing, London, UK. 100-136.

Hoffmann, J. P. (1998). Wastewater treatment with suspended and nonsuspended algae. *J Phycology,* **34**, 757–763.

Jiménez-Pérez, M.V., Sánchez-Castillo, P., Romera, O., Fernández-Moreno, D., and Pérez-Martınez, C. (2004). Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure. *Enzyme and Microbial Technology*, **34**, 392–398.

Mayo, A.W. (1995). Modeling coliform mortality in waste stabilization ponds. *Journal of Environmental Engineering ASCE*, **121**, 140-152.

Megharaj, M., Pearson, H.W. and Venkateswarlu, K. 1992. Removal of nitrogen and phosphorus by immobilized cells of Chlorella vulgaris and Scenedesmus bijugatus isolated from soil. *Enzyme, Microbiology and Technology*, **14**, 656-658.

Moreno-Garrido, I (2008). Microalgae immobilization: Current techniques and uses. *Bioresource Technology* **99**, 3949–3964.

Pearson, H.W., Mara, D.D. and Arridge, H. A. (1995). The influence of pond geometry and configuration on facultative and maturation waste stabilization pond performance and efficiency. *Water Science and Technology*, **31**(12), 129-139.

Shilton, A. and Sweeney, D. (2005). Hydraulic Design. *In Pond Treatment Technology*, Shilton (edit). IWA publishing, London, UK. 188-217.

Tam, N.F.Y. and Wong, Y.S. (2000). Effect of immobilized micro-algal bead concentrations on wastewater nutrient removal. *Environmental Pollution*, **107**, 145-151.

von Sperling, M. (1999). Performance evaluation and mathematical modeling of coliform die-off in tropical and subtropical waste stabilization ponds. *Water Research*, **33**, 1435-1448.